Surface carbohydrates of the milk fat globule membrane.

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SURFACE CARBOHYDRATES OF THE MILK FAT GLOBULE MEMBRANE

Submitted by

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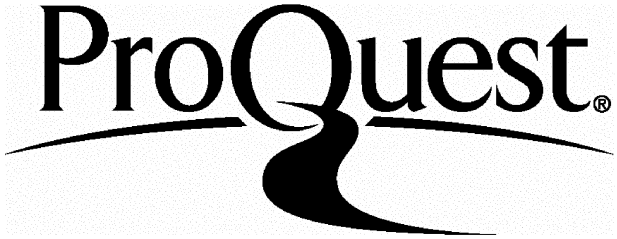

To my parents and family

for their patience and

support

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SUMMARY

Treatment of bovine milk fat globules with pronase resulted in the release of a soluble sialoglycopeptide fraction which was chromatographed on Sephadex G-50 as a single peak. Fractionation of this fraction by ion-exchange chromatography allowed the separation of two sialoglycopeptides; the sialic acid-poor (SP) and the sialic-acid-rich (SR) glycopeptides. Both fractions were found to be homogeneous by SDS-PAGE and by gel filtration on Sephadex G-75. Antisera were raised against both fractions and their contribution to the antigenicity of the bovine milk fat globule membrane (MFGM) was briefly studied. The carbohydrate composition of both glycopeptide fractions was determined by gas liquid chromatography. Preliminary amino acid analysis showed that the major amino acids in the SR fraction were serine and threonine whereas the dominant amino acid in the SP fraction was found to be asparagine.

The accessibility of specific carbohydrate residues on the surfaces of intact and neuraminidase treated bovine and human milk fat globules was compared by means of lectin-based agglutination assay and by the use of the Fluorescence-Activated Cell Sorter. The presence of galactose, mannose and/or glucose and N-acetyl-glucosamine, but not fucose was detectable on both bovine and human globules. The major differences between bovine and human milk fat globules were the presence on human globules of high amounts of *Arachis hypogaea* receptor (Thomsen-Freideriech antigen), none of which was apparently masked by sialic acid and the presence of unsubstituted N-acetylgalactosamine on bovine but not on human milk fat globules.

Pooled bovine, but not human, milk fat globules were found to be agglutinated by relatively high dilutions of the K99 adhesin (antigen) isolated from E. coli B41. Parallel studies of K99-induced agglutination of bovine globules and sheep erythrocytes were carried out by using bovine MFGM-derived sialoglycopeptides and a range of monosaccharides as specific inhibitors. The results suggest that N-acetylgalactosamine-containing carbohydrate complexes are the most likely candidates for the K99 receptor on the surfaces of bovine globules and sheep erythrocytes.

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Section C

Investigation of exposed receptors for the K99 adhesin on the surfaces of bovine milk fat globules and sheep erythrocytes

The development of ideas on membrane structure

Early ideas about the composition and structure of biological membranes were based upon what could be gleaned from histological examination under the light microscope and what could be deduced from their functions. More accurate knowledge of their make-up depended upon the development of suitable methods of isolation coupled with the discovery of more specific histochemical techniques. In fact the cell membranes were not actually seen until the introduction of the electron microscope, which revealed not only the plasma membrane bounding the cell but also many membranes within it. The development of ideas on the structure of biological membranes has been dealt with in a number of reviews (Robertson, 1964; Singer, 1971, 1977; Nicolson, 1976; Harrison and Lunt, 1980). In this section the historically-most important models of biological membranes will be briefly discussed.

I. The Danielli-Davson-Robertson Model

In 1925 Gorter and Grendel, from their studies on acetone extracts of erythrocyte membranes from a variety of species, concluded that the area occupied by the membrane lipids in the Langmuir trough was approximately double that of the surface area of the intact erythrocytes. They pointed out that this is consistent with the presence in the erythrocyte membrane of a lipid bilayer in which the hydrocarbon chains all occupy the centre of the bilayer and the polar head groups face outward as shown in Fig. 1. The work of Gorter and Grendel was later criticised by Winkler and Bungenberg (1941) and by Hoffman (1962), who suggested that the erythrocyte lipids not only were incompletely extracted but that the erythrocyte surface area was underestimated. Davson (1962) pointed out that the errors in Gorter and Grendels' work fortuitously cancelled each

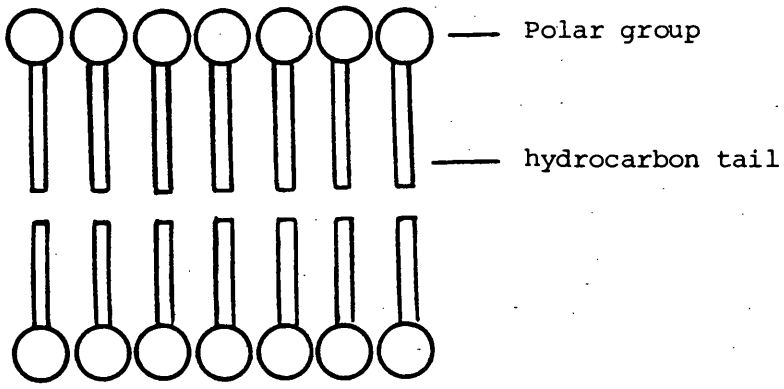


Fig. 1. After Gorter and Grendel (1925)

other out to give a working model system. It is now well known that sufficient lipid is indeed available to cover the erythrocyte surface completely as a bilayer, but only if that bilayer is highly expanded.

The presence of proteins (Fig. 2) in biological membranes was first proposed by Danielli and Davson (1935) in order to explain what seemed to be anomalously low surface tensions of biological membranes compared with those of model lipid systems. This model formed the basis of membrane structure for over thirty years although it was subsequently modified by Danielli (1958) to include protein-lined pores which penetrated the lipid bilayer and so allowed to the possibility of transmembrane permeability (Fig. 3).

The fact that electron micrographs of membranes from a variety of sources exhibited a characteristic trilayer appearance led Robertson (1964) to propose the concept of a universal 'unit membrane'

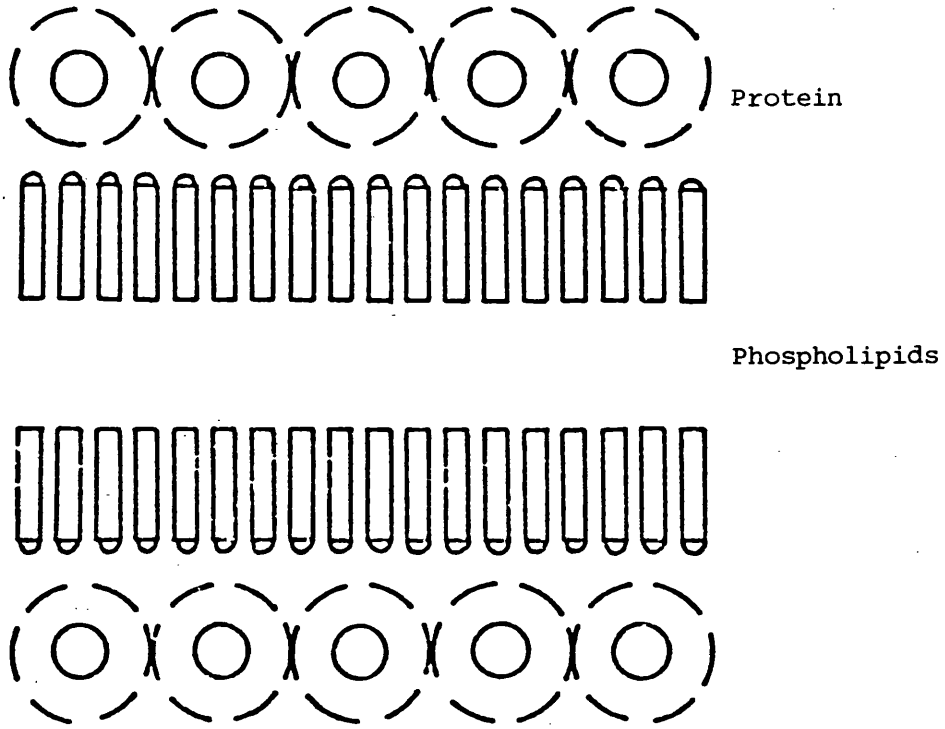


Fig. 2. After Danielli and Davson (1935).

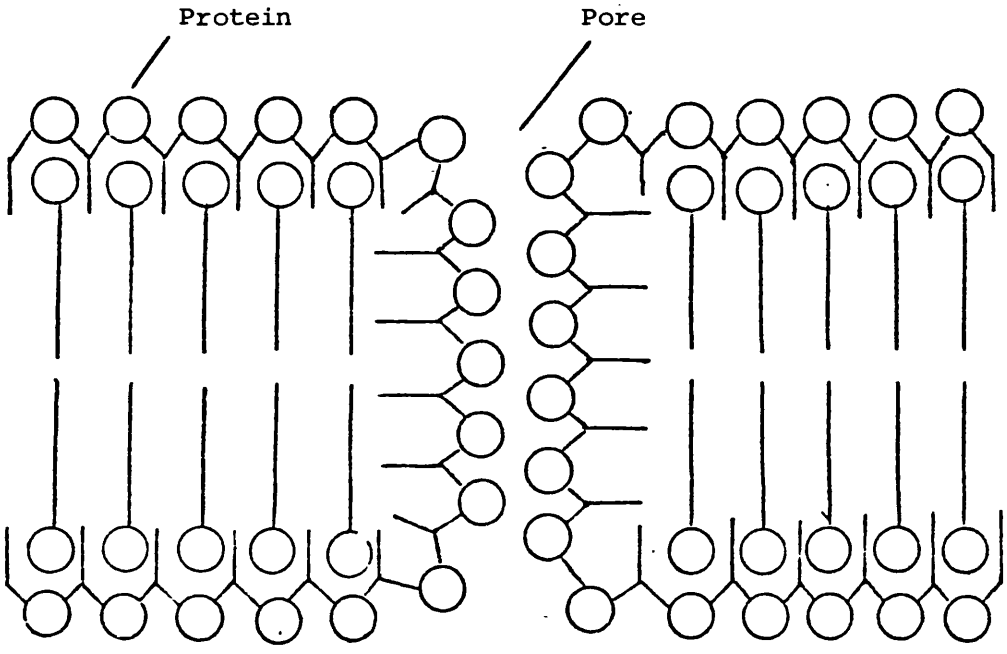


Fig. 3. After Danielli (1958).

based on the Danielli-Davson model. Because of the findings in the early 1960s that the plasma membrane was an asymmetric structure, Robertson (1964) modified the model of Danielli and Davson by introducing an asymmetric distribution of protein molecules about the lipid bilayer. (Fig. 4). Although this model was supported by the electron microscopic appearance of biological membranes, particularly myelin, it has since been realised to be inadequate in a number of respects. Thus,

(i) It bases a general model for membrane structure on the electron microscopic observation of myelin. This led Korn (1966) to emphasise that myelin is not typical of membranes as a whole, particularly with respect to its lipid content which is 80% of the dry weight of the membrane compared with about 50% for most membranes.

(ii) The trilaminar patterns of 'typical membrane electron micrographs' require the use of tissue fixing reagents, whose mode of action is not clearly understood and indeed electron micrographs remarkably similar to those of natural membranes have been obtained from artificial lipid bilayers containing no protein (Palmier and Hall, 1972).

(iii) The model is inherently inflexible in explaining the obvious variations in composition and function of membranes from various sources.

(iv) Optical activity data indicate that membrane proteins are globular proteins containing 25 - 40% α-helical structure (Urry et al., 1970; Urry, 1972; Zahler et al., 1972) and not β-sheets as required by the dimensional restrictions

of the Danielli-Davson-Robertson model (Vandenheuvel, 1965).

(v) The Danielli-Davson-Robertson model predicts that ionic head groups of phospholipids would be shielded from contact with the aqueous phase by the polar groups of the protein monolayers. This burying of polar groups in a non-polar environment is energetically unfavourable.

Further discussion of these points can be found in Singer (1971) and Harrison and Lunt (1980).

II. Subunit Models

As the methods of tissue sectioning and electron microscopy improved, the discontinuities in the tramline appearance of sectioned membrane became more apparent. A speculative suggestion at the time by Lucy (1964) was that phospholipid could also exist in a micellar form, either entirely or in equilibrium with the bilayer, thus explaining the discontinuous patterns observed.

Evidence from mitochondrial membrane preparations indicated that the membrane might be constructed from repeating globular units, which led Sjostrand (1968) to suggest a subunit arrangement of structural membrane components based on the suggestion of Lucy, but with protein filling the space between the lipid micelles (Fig. 5). A similar model of Green et al. (1967) proposed that membranes are made of repeating cuboidal units which interact to form a membranous sheet. This model was again based on mitochondrial membranes although it was vigorously promoted as a general structure in membranes.

The subunit models based on electron microscopic phenomena and biochemical studies of mitochondrial inner membranes, were criticised

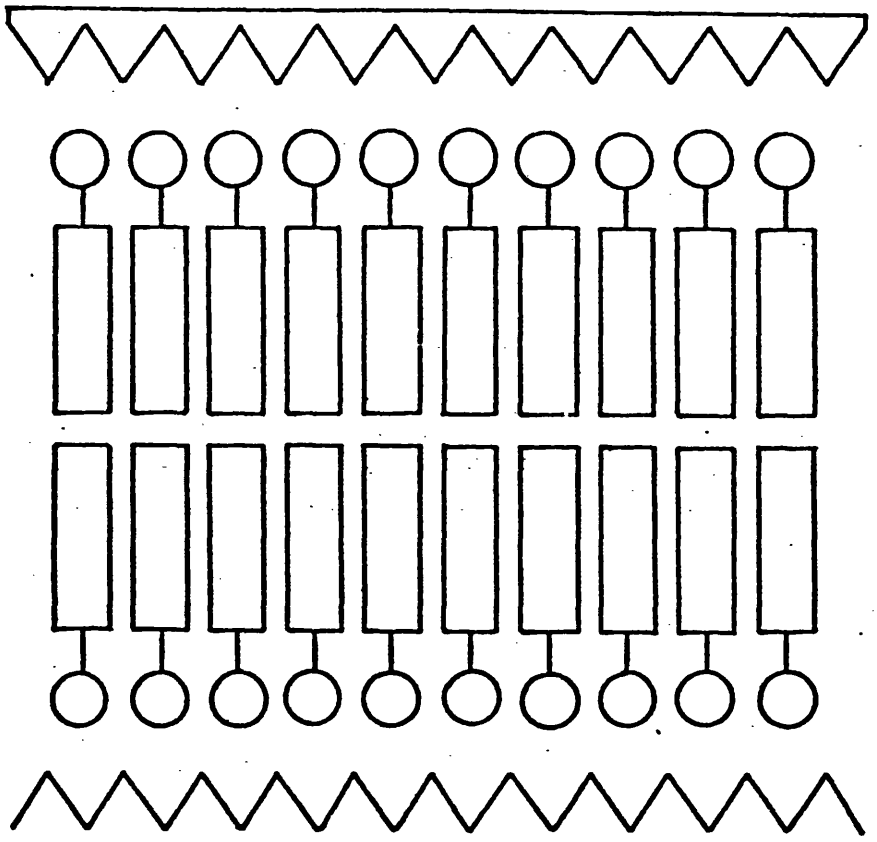


Fig. 4. After Robertson (1964).

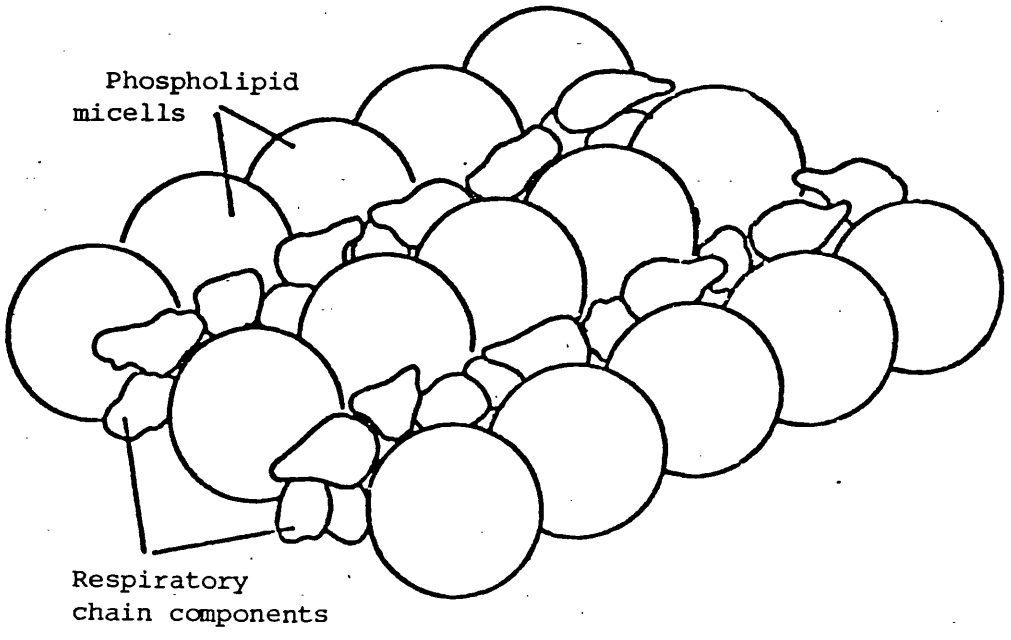


Fig. 5. After Sjöstrand (1968).

mainly because of:

(i) Mitochondrial membranes are exceptional in having a high content of protein (80%) and a strong requirement for structural organization.

(ii) X-Ray diffraction studies on the membrane did not show any indication of globular substructures (Thompson et al., 1968) which were accordingly suggested to be artefacts.

III. Mosaic Models

Evidence that both proteins and lipids were exposed on the surface of cell membranes led Wallach and Zahler (1966) and Lenard and Singer (1966) to propose a model involving a mosaic arrangement of the two components in the membrane. As shown in Fig. 6, this model involves globular proteins embedded in, and occasionally crossing, a lipid bilayer core. In such a structure the polar groups oflipid and protein are in direct contact with the aqueous surroundings. while the non-polar residues of both molecular species are sequestered in the heart of the structure, away from water. This model satisfies the physico-chemical data and offers a thermodynamically sound alternative to the earlier models as well as allowing a potential route through the lipid bilayer barrier by way of the transmembrane protein. In contrast to the Danielli-Davson-Robertson model, which requires that the protein on the surface be predominantly in the β-conformation, the mosaic model proposes that membrane protein exists as globular protein which commonly contains proteins of α-helix and random coil conformation in accord with optical evidence. Some of these globular proteins are seen as penetrating the lipid bilayer whereas others are arranged on the surface bounding the lipid core. The idea of two classes of

membrane proteins agrees with experimental findings that some proteins [integral (Singer, 1971) or intrinsic (Vanderkooi, 1972) proteins] can only be extracted by agents causing extensive disruption of the membrane. A second class of proteins known as peripheral (Singer, 1971) or extrinsic (Vanderkooi, 1972) proteins are characterised by being dissociated from membranes by mild treatments such as increased ionic strength or chelating agents. In contrast to integral proteins, they can be obtained in soluble form in simple aqueous solutions.

Enzymic modification of the lipid components of erythrocyte membranes has indicated that the lipid bilayer is, indeed, interrupted by non-lipid components to the extent of 30% (Finean et al., 1971; Coleman et al., 1970). Evidence that some proteins actually cross the lipid bilayer was provided by Bretscher(1971) who used the membrane-impermeable reagent [^{35}S]-FMMP (formyl methionyl sulphone methyl phosphate) to label membrane proteins in both intact erythrocytes and erythrocyte ghosts. Certain proteins were found to be doubly-labelled following extraction from ghosts but not from intact membrane. These findings led Bretscher to conclude that two major proteins, of molecular weights 105,000 and 90,000 respectively, span the membrane. These conclusions are generally accepted, although it could be argued that labelling of certain polypeptides only in ghosts arises from reorganisation of such proteins during the preparation of the ghosts rather than from their location on the inside of the membrane. Evidence was also provided from SDS-electrophoretic patterns of the major erythrocyte protein (band 3 polypeptide) (Jenkins and Tanner, 1975; Robert et al., 1977). More obvious demonstrations of the existence of proteins embedded in the membrane comes from freeze-etching or freeze-fracture electron microscopy. In this procedure, a fresh membrane specimen is

rapidly frozen under vacuum and fractured with a microtome knife. Frozen water is then sublimed (freeze-etched) from the exposed surface, which is metal shadowed and replicated. Electron microscopy of the surface replica reveals the topography of the interior hydrophobic exposed face. Such studies on, for example, the erythrocyte membrane (Pinto da Silva and Branton, 1970; Pinto da Silva and Nicolson, 1974), the chloroplast lamellae (Branton and Park, 1967), and the retinal rod outer segment (Clark and Branton, 1968) provide visual evidence of protein molecules apparently crossing the mid-plane of the lipid bilayer of the membrane.

As specific membrane proteins have become better characterised, it has become possible to demonstrate that some integral proteins possess hydrophobic amino acid sequences which apparently correspond to those sections of the polypeptide that cross the lipid bilayer. Thus, amino acid sequences of this type have been demonstrated, in glycophorin A of human erythrocyte membrane (Marchesi et al., 1972) and in cytochrome b_5 (Ozols, 1972). The lipoprotein of E. coli outer membrane is particularly interesting in that its hydrophobic amino acids are so arranged that in an α-helical form, the peptide has a hydrophobic and hydrophilic face. Aggregation of several such helices could provide a hydrophilic ion-transporting channel encased in a hydrophobic exterior in contact with the lipid bilayer. A model of this type shown in Fig. 7 demonstrates the self-association of integral membrane glycoprotein molecules to from hydrophilic trans-membrane channels (Hughes, 1975). In this case, the pore diameter generated by self-assembly of six α-helical subunits (1 nm) would probably be sufficient for the transport of small hydrated ions,

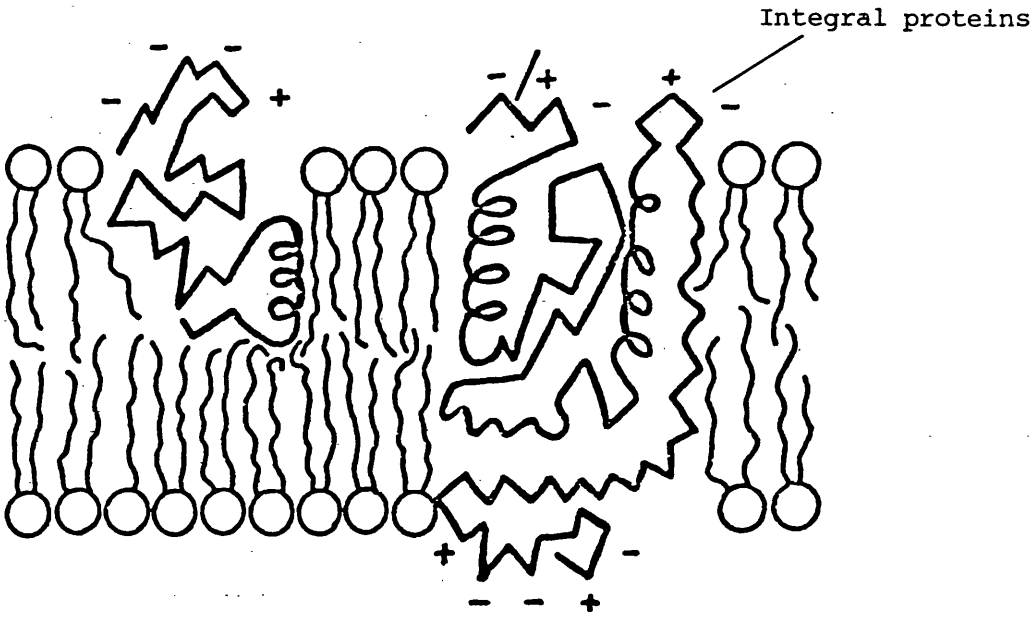


Fig. 6. After Lenard and Singer (1966).

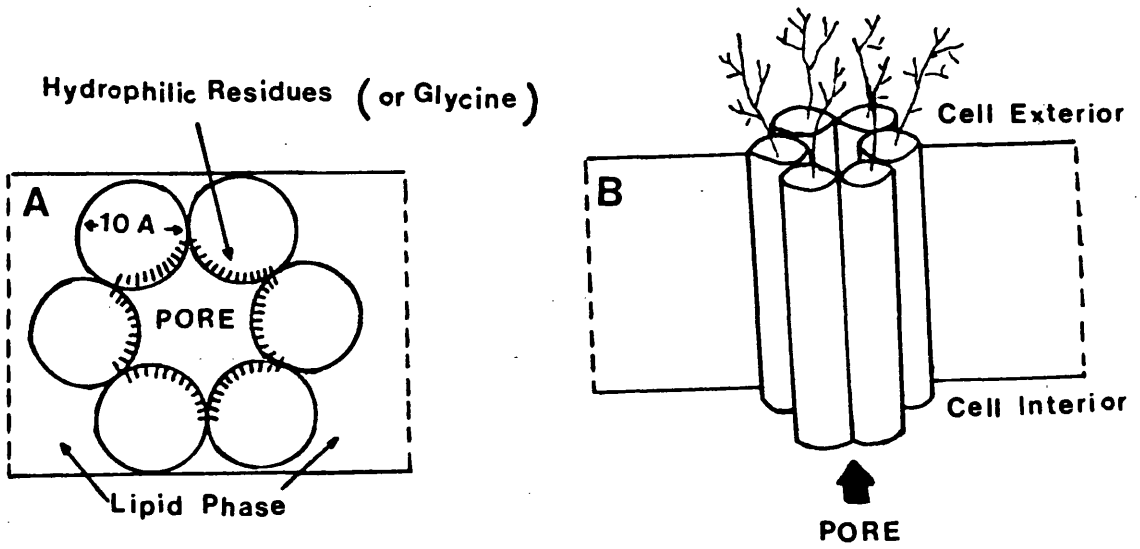


Fig. 7. Hypothesis for generation of water filled channels by self assembly of glycoprotein subunits, (a) the assembly of six helical peptide segments projects a hydrophilic face to the inside of the pore, (b) side view of the assembly showing the polypeptide spanning the lipid bilayer of the membrane and the carbohydrate chains of the glycoprotein subunits extending to the external space (after Hughes, 1975).

nutrients or even larger extended molecules and could, of course, be considerably greater than this if higher aggregates are formed.

Aggregation of membrane proteins implies lateral motion within the plane of the membrane and indeed the mosaic model was amplified by Singer and Nicolson (1972) when they stressed the dynamic aspects of membrane structure in describing the fluid mosaic model (Fig. 8). The fluid mosaic model sees the membrane as a solution of proteins in a two-dimensional viscous solvent system of phospholipids,the bulk of which is organised as a discontinuous fluid bilayer. By introducing the fluidity into the mosaic model some of the previously unexplained membrane phenomena could be clarified. For example, deviations from the electron microsopic tramline appearance of sectioned membrane preparations, in regions of membrane specialisation such as tight and gap junctions (McNutt and Weintein, 1973) can be explained in terms of local reorganisation of intercalating membrane components. The fluid mosaic model is essentially a dynamic one and considerable emphasis is placed on the diffusion of proteins within the lipid matrix, which is assumed to be predominantly in a bilayer form. The studies of Chapman et al. (1967), using nuclear magnetic resonance (n.m.r.) indicated that the molecular motion of individual carbon atoms along the fatty acyl chains of phospholipids increases towards the interior of the membrane giving the whole a viscosity resembling that of a light oil. Sharp signals obtained from choline, sialic acids and neutral sugars indicated that these groups are relatively unconstrained on the exterior of the membrane. Electron-spin resonance (e.s.r.) studies of Kornberg and McConnell (1971) and Scandella et al. (1972) indicated that phospholipid molecules move rapidly in the plane of the membrane. Integral membrane

proteins have also been shown not only to rotate about an axis perpendicular to the plane of the membrane (Brown , 1972; Cone, 1972) but also to diffuse laterally within the plane of the membrane. This was first demonstrated by Frye and Edidin (1970) who reported the random mixing of fluorescently-labelled cell surface and antigens following the virally-induced formation of mouse-human heterokaryons. Labelled antibodies were also used in similar experiments on muscle fibre surface antigens (Edidin and Fambrough, 1973). By following the redistribution of rhodopsin molecules after bleaching of part of the retinal rod membrane, Poo and Cone (1974) established a lateral diffusion coefficient for rhodopsin of 3.5×10^{-9} cm^2/S. E.s.r., n.m.r. and more recently, fluorescence techniques have all been applied to measure the lateral diffusion coefficients (D) of lipid probes, and similar values of D (of the order of 10^{-8} cm^2/s) have been found both in a range of cell membranes and, above the phase transition, in model phospholipid bilayer systems (Devaux and McConnell, 1972; Brown, 1972; Cone, 1972; Poo and Cone, 1974; Koppel *et al.* 1976). Tayler *et al.* (1971) studied the effects produced on lymphocytes by the addition of divalent antibodies directed to their surface immunoglobulin molecules. The antibodies induce a redistribution and pinocytosis of these surface immunoglobulins, so that within about 30 minutes at $37^{o}C$ the surface immunoglobulins are completely swept out of the membrane. The changes in the distribution of the surface immunoglobulins (receptors) involve firstly the formation (metabolism-independent) of clusters and patches of receptors and secondly the emergence (metabolism-dependent) of a 'cap' of aggregated receptors over one pole of the cell (Fig. 9). Clustering of the intramembraneous particles has also been observed to accompany the agglutination

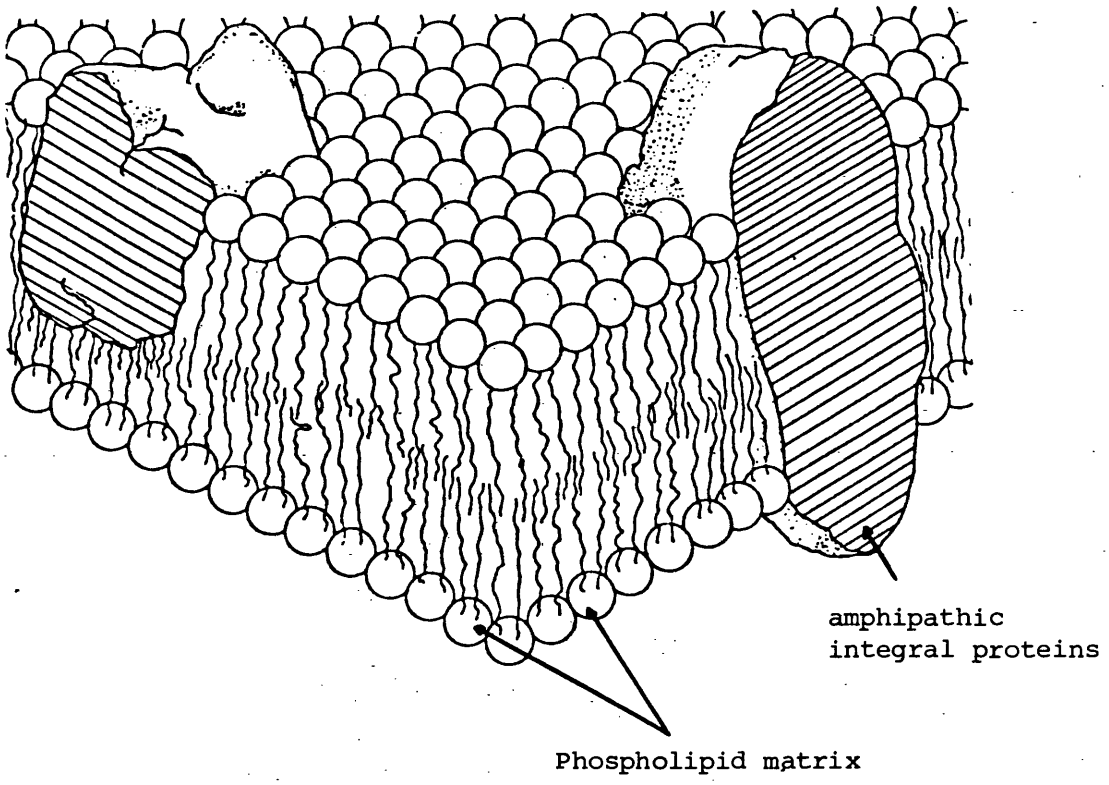


Fig. 8. After Singer and Nicolson (1972).

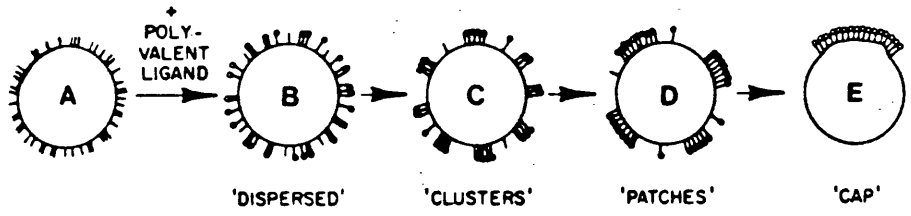


Fig. 9. Stages resulting from the interaction of a polyvalent ligand with surface receptors on a lymphoid cell (After Nicolson, 1976).

of mouse plasmacytoma cells by concanavalin A agglutinin (Benedetti et al., 1973). It has been reported that polymeric antigens can 'cap' lymphocyte receptors in the same way (Diener and Paetkau, 1972; Raff et al., 1973; De Luca, 1975). This aggregation of surface receptors in the plane of the membrane can clearly only occur if the receptors are free to diffuse in the membrane. The phenomenon of 'capping' suggests moreover that movement of integral membrane proteins can be a concerted process subjected to overall direction. In this context the finding of Nicolson and Painter (1973) that the binding of specific antibodies to spectrin on the cytoplasmic side of the erythrocyte ghost membrane causes a redistribution of sialyl groups (of glycoproteins) on the outer side of the membrane is of significance. This suggests that extrinsic proteins may control the distribution and mobility of intrinsic transmembrane components which have their prime activity at the external face of the membrane structure. It is possible that extrinsic proteins can also act to restrict the motion of intrinsic proteins and indeed the red cell has been noted as unusual in that, unlike many other mammalian cells, antigen-antibody complexes on its surface often fail to clump in the plane of the membrane. The interaction of the intrinsic transmembrane antigen with spectrin on the cytoplasmic face of the membrane could explain this. Although spectrin is unique to the erythrocyte membrane most mammalian cells have an analogous cytoplasmic protein network composed of microfilaments and microtubules. On the basis of the above and other data Edelman and Yahara (1973, 1974, 1975a, b) proposed a model to account for receptor diffusion in the plane of the membrane involving transmembrane control by microtubule - microfilament systems (Fig. 10). In this model, the function of microfilaments

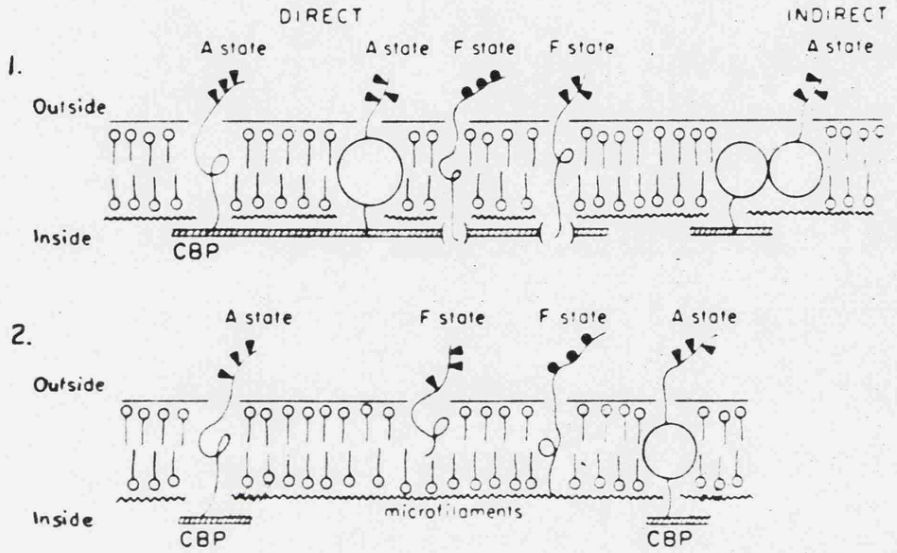


Fig. 10. Transmembrane control by membrane-associated cytoskeletal elements as proposed by Edelman (1974). Receptors are assumed to interact with colchicine binding proteins (CBP) in an equlibrium consisting of two states, anchored (A) and free (F). As indicated in (1), receptors may interact directly or indirectly via the membrane-intercalated particles. Alternatively, as shown in (2), only certain glycoprotein receptors may interact with the CBP and the CBP may interact with membrane associated microfilament assemblagens.

is envisaged as being mainly contractile, serving to direct the movement of receptors within the plane of the membrane, whereas that of microtubules is skeletal, maintaining a network of membrane 'anchorage' points which may restrict or release surface receptor depending on both extra- and intracellular stimuli. The details of this cytoskeletal control system are far from clear but currently a

great deal of research is being devoted towards clarification of its role in controlling a range of cellular functions including cell adhesion, motility and response to extracellular agents generally. It is likely that significant advances in biochemical understanding of the cell membrane will come from this aspect of its function in the near future.

Membrane glycoproteins

Glycoproteins make up a large and heterogeneous group of macromolecules and serve a variety of different functions. They occur as soluble secreted molecules such as plasma glycoproteins, protein hormones, immunoglobulins, mucins, blood group substances, acid mucopolysaccharides (glycosaminoglycans) and also in collagens and basement membranes. Current interest in membrane structure has drawn the attention of many researchers to the fact that glycoproteins also occur in an insoluble form as components of cell membranes. The fluid mosaic model of membrane structure (Singer and Nicolson, 1972) emphasized the occurrence of integral proteins, the structure of some of which have recently been studied, and shown to include carbohydrate. Some membrane glycoproteins have been isolated by extraction of the membrane with dissociating reagents (Juliano, 1978) while the presence of others has been detected by more indirect methods such as the use of lectins (Lotan and Nicolson, 1979), cell electrophoresis (Pertlow and Pertlow, 1979) and cytochemical reagents (Juliano, 1978).

Cytochemical techniques such as those involving the periodic acid-Schiff (PAS) reagent (Leblond, 1950) have shown carbohydrate to be a characteristic feature of all cell surfaces except in the region of junction complexes (Rambourg et al., 1966). The PAS stain for carbohydrate depends on periddate oxidation of carbon-carbon bonds bearing vicinal hydroxyl groups to give carbonyl groups. These will react with Schiff's reagent (leucofuchsin) yielding a pink or purple Schiff's Base. Carbohydrates staining with PAS is always asymmetrical in that the stain is found to be distributed on the

external surface but not on the cytoplasmic surface of the plasma membrane (Rambourg and Leblond, 1967).

Electron-dense markers such as colloidal iron (Gasic and Berwick, 1962; Benedetti and Emmelot, 1967), colloidal thorium (Rambourg and Leblond, 1967), ruthenium red (Luft, 1971) and cationized ferritin (Danon et al., 1972; Hakenbrock and Miller, 1975) have been used to demonstrate the presence of acidic or anionic sites on the cell surface. These reactions are usually attributed to the presence of carboxyl groups of sialic acid residues of integral membrane proteins exposed at the external surface of the cell (Winzler, 1969). As is the case for PAS staining, carbohydrate is not normally detected by these means on the cytoplasmic face of the plasma membrane although anionic binding sites have been detected by cationised ferritin on the outer (cytoplasmic) face of the Golgi complex (Abe et al., 1976). However, the membrane-associated carbohydrates on the cytoplasmic surface are of much lower density than those of the cisternal membrane face. Concanavalin A has also been used to detect surface carbohydrates (mannose) residudes and has been shown to bind not only to the cisternal surface but also, to a limited extent, to the cytoplasmic surface of Golgi membranes (Abe et al., 1977). This suggests that carbohydrate residues are present, and probably assembled, mainly on the inner aspect of the membrane, but that some may also be exposed on the cytoplasmic surface.

In view of the possible involvement of carbohydrate in some of the biological functions displayed by glycoproteins, a great deal of effort has been extended in recent years to determine the structure

of the oligosaccharide chains of both soluble and membrane glycoproteins. The sugars present in the carbohydrate portion of membrane glycoproteins are few and include D-galactose, D-mannose, D-glucose (rarely) L-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and sialic acid, arranged in oligosaccharide side chains of varying sizes and glycosidically-linked to a polypeptide backbone. It has become customary to classify glycoprotein oligosaccharide structures on the basis of the nature of the carbohydrate-peptide linkage. This approach has been useful because the carbohydrate-amino acid linkage dictates the properties of the associated oligosaccharide group and because a single glycoprotein molecule may contain oligosaccharides of more than one linkage type. On this basis two major types of oligosaccharide complex have been shown to occur in glycoproteins. The first of these involves an N-glycosidic bond from N-acetylglucosamine to the amide nitrogen of asapragine (Fig. 11) which is relatively stable to hydrolysis by strong alkali. The second type of linkage contains an O-glycosidic bond from N-acetylgalactosamine to the hydroxy group of serine or threonine (Fig. 11) and can be cleaved by mild alkaline treatment which promotes a β-elimination reaction (Fig. 13). This difference between N-glycosidic and O-glycosidic linkages has been exploited to advantage in the detection and separation of the two types of oligosaccharide chains.

The structures of some glycopeptides which contain O-glycosidic linkages involving N-acetylgalactosamine as the linkage sugar are shown in Table 1. N-glycosidically-linked (to aspragine) oligosaccharide units are usually found to be of two types. The first contains only mannose and N-acetylglucosamine and is called

	Structure	Glycoprotein
A	NANA $\xrightarrow{\alpha 2.6}$ GalNAc $\longrightarrow$ Ser(Thr) (NGNA)	Submaxillary mucins
B	Gal $\xrightarrow{\beta 1.3}$ GalNAc $\longrightarrow$ Ser(Thr)	"Antifreeze" glycoprotein of antarctic fish: Human IgA1; β subunit HCG: cartilage keratine sulfate; epiglycanin of TA_3.HA cells; lymphocyte, RBC, and milk fat globule membranes
C	Gal $\xrightarrow{\beta 1.3}$ GalNAc $\longrightarrow$ Ser(Thr) $\uparrow\alpha 2.3$ NANA	Bovine kininogen; epiglycanin of TA_3-HA cells; B_{16} melanoma cells
D	Gal $\xrightarrow{\beta 1.3}$ GalNAc $\longrightarrow$ Ser(Thr) $\uparrow\alpha 2.3$ $\uparrow\alpha 2.6$ NANA NANA	Fetuin; human RBC membrane sialoglycoprotein; bovine kininogen; rat brain
E	Gal $\xrightarrow{\beta 1.3(4)}$ GlcNAc $\xrightarrow{1.2(4.6)}$ Gal $\xrightarrow{\beta 1.3(4)}$ GalNAc $\longrightarrow$ Ser(Thr) $\uparrow\alpha 2.3$ NANA	Epiglycanin
F	Gal $\xrightarrow{1.3}$ GlcNAc $\xrightarrow{1.3}$ GalNAc $\longrightarrow$ Ser(Thr) $\uparrow 1.6$ GlcNAc $\uparrow 1.4$ Gal	Human gastric mucin; core region of human and hog blood group substances

Table 1. Glycopeptides linked through N-acetylgalactosamine to the hydroxyl group of serine and threonine (after Kornfeld and Kornfeld, 1980).

"high-mannose" or 'simple' type (Fig. 12). The second contains a variable number of outer chains linked to a β-mannosyl-di-N-acetyl-chitobiose unit, the same structure that occurs in the inner region of typical high-mannose oligosaccharides (Fig. 12).

Detailed information about the isolation and structural characterisation of membrane glycoproteins and glycopeptides can be found in a number of recent reviews (Sturgess et al., 1978; Tanner, 1978; Kornfeld and Kornfeld, 1980).

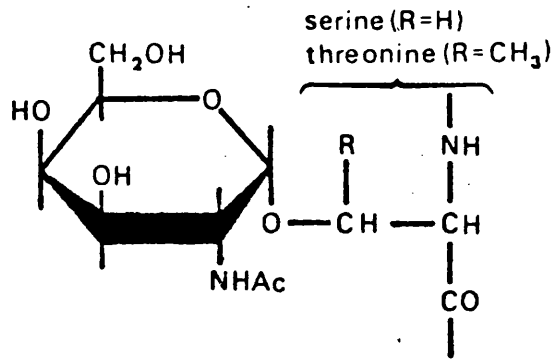


Fig. 11a. O-glycosidic linkage to serine or threonine.

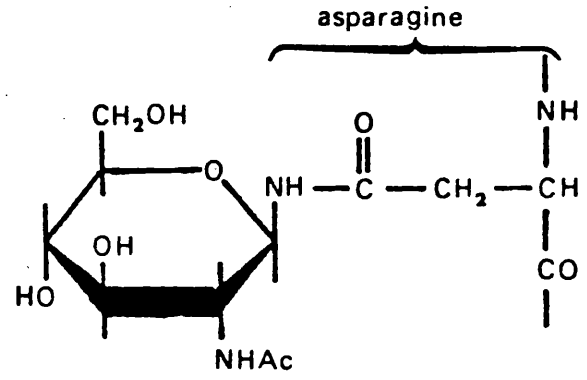


Fig. 11b. N-glycosidic linkage to asparagine.

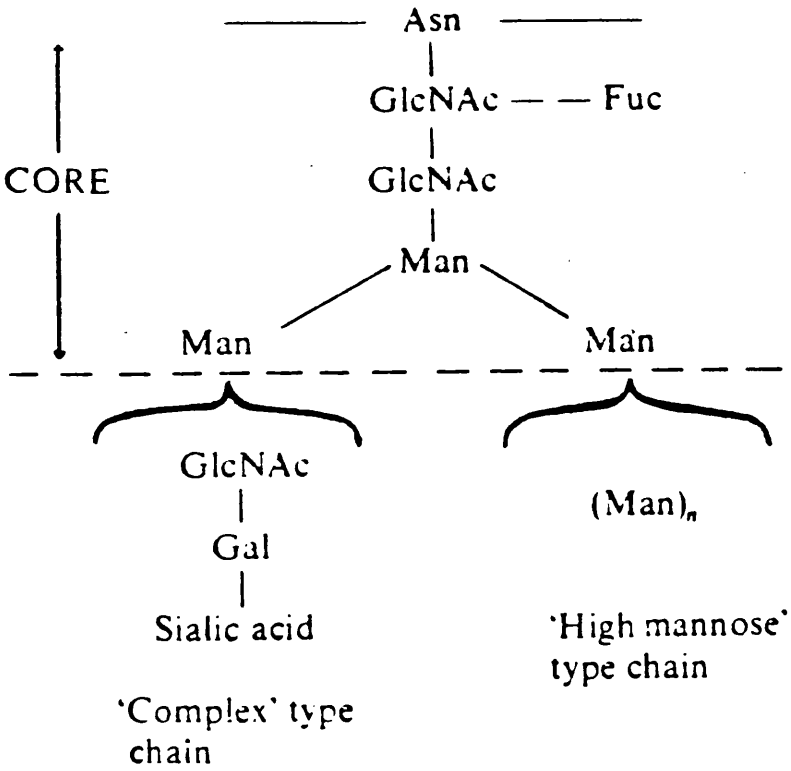


Fig. 12. Schematic representation of a typical N-glycosidically-linked oligosaccharide complex in secreted glycoproteins. After J. Montreuil (1975).

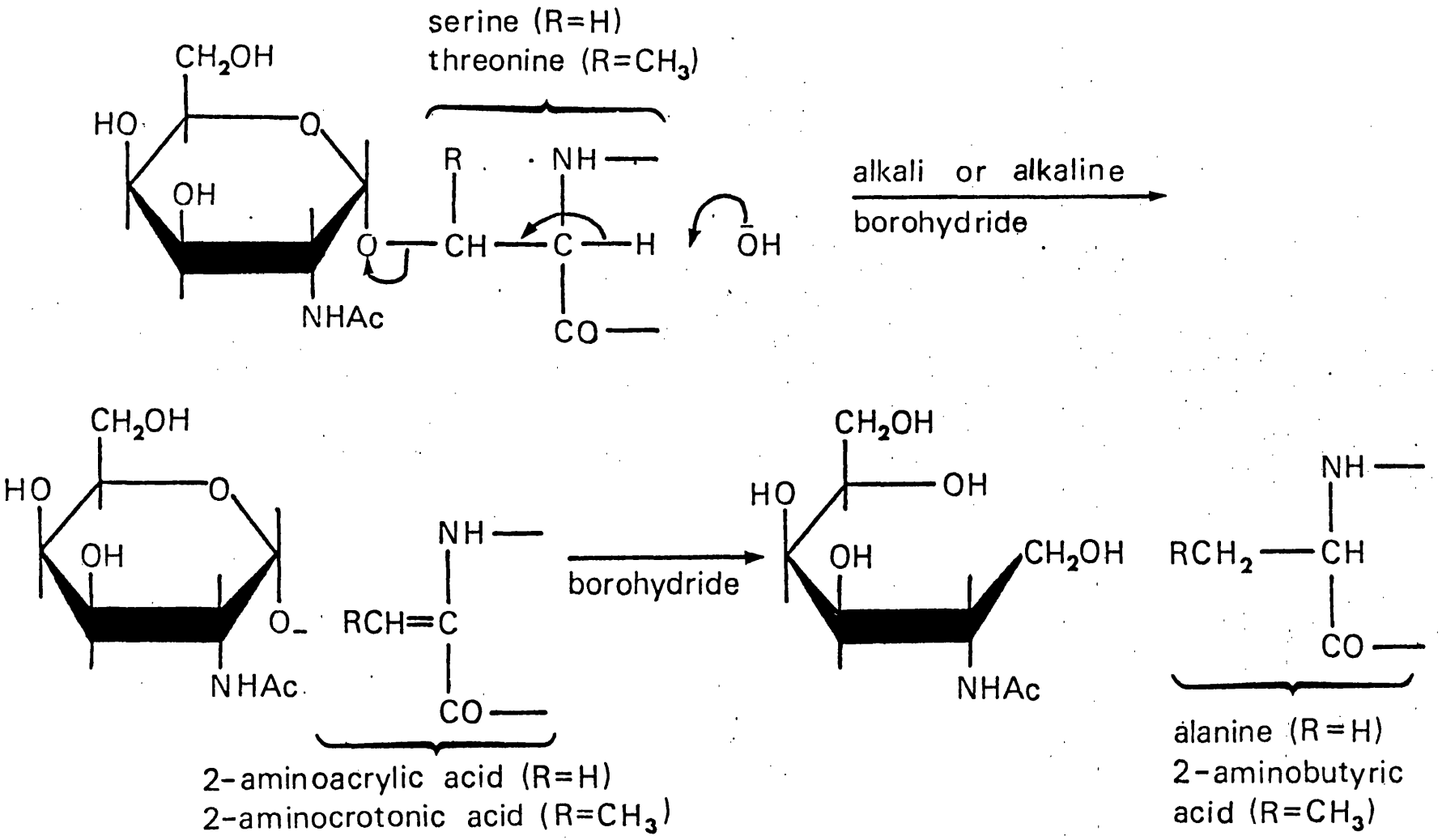


Fig. 13 Scheme for alkaline borohydride reduction.

Membrane carbohydrates as specific determinants

In view of the presence of complex glycoprotein and glycolipid molecules at the outer surface of animal cells, it might be expected that these molecules are involved in the interaction of the cells with their surroundings. The sugar complexes are excellent candidates for participating in the various specific functions of the cell membrane. With a basic set of seven monosaccharides (fucose, glucose, galactose, mannose, N-acetylglucsamine, N-actylgalactosamine and sialic acid) the number of possibilities of structural variations in the oligosaccharide complexes of glycosubstances is potentially very great. There are, for example 10^{24} possible structures for a twelve-residue oligosaccharide containing three mannose, three galactose, three N-acetylgucosamine and three sialic acid residues, and 2^4 possible structures for only two hexose units, glycosidically linked to protein, assuming that both monomers are pyranose rings.

Cell-surface-expressed carbohydrates have indeed been found to participate in a range of recognition phenomena including blood group specificity (Watkins, 1974; Hakomori, 1981; Anstee, 1981), hormone reception (Kohn et al., 1978) and bacterial adhesion (Ofek et al., 1978).

(i) The ABH(O) blood group system

Antigenicity expressed through carbohydrate determinants is best established in the ABH blood group system (Watkins, 1972). Formulation of the relationship between the sequence and linkages of carbohydrates and their ABH and Lewis antigenic specificity was established as a result of the work of Morgan and Wakins and of Kabat and associates during the 1950s and the early 1960s. As a

result of this work it has become established that a precursor-end-product relationship exists between related antigens, e.g., between H and A (or B) or between Le^a and Le^b, and that the glycosyltransferases that catalyse these interconversions are the primary gene products (Marcus, 1969; Watkins, 1966, 1972). Although it has long been generally understood that the ABH determinannts expressed at the surface of erythrocytes are components of membrane glycolipids (Hakomori and Strycharz, 1968; Gardas and Koscielak, 1974), several workers (Whitemore et al., 1969; Liotta et al., 1972; Hamaguchi and Cleve, 1972; Gardas and Koscielak, 1973) have reported evidence for the presence also of ABH antigenically-active glycoprotein in human erythrocyte membranes. These conclusions were contested by Anstee and Tanner (1974 a,b), who ascribed the ABH activity of membrane-derived water-soluble glycoprotein fractions to tightly bound glycolipid molecules or to glycolipids with unusual solution properties. Nevertheless more recent advances in chemical and analytical methods in membrane biology have resulted in the validation of much of this work and, a relatively clear picture has now emerged showing that blood group ABH and Ii determinants are carried not only by glycolipids, but also by some membrane glycoproteins. It is now apparent that four classes of carbohydrate chains carry ABH and Ii blood group determinants, these are:

(i) Simple glycolipids containing 5 - 10 glycosyl residues (Koscielak, 1978).

(ii) Polyglycosylceramides containing up to 30 glycosyl residues (Koscielak, 1978).

(iii) Alkali-stable oligosaccharide chains carried on band 3 and band 4.5 glycoproteins (Krusius et al.,1978; Järnfett et al., 1978).

(iv) Alkali-labile oligosaccharide chains probably carried on glyophorin (Takasaki et al., 1978).

The oligosaccharide determinannts contributing to human ABO blood group antigenicity are shown in Fig.14. The current state of knowledge concerning the ABO and the related Ii antigens of human erythrocytes has been recently reviewed by Hakomori (1981).

(ii) The MNS blood group system

The MN antigens, the second major blood group system described by Landsteiner and Levine (1928) , are believed to be controlled by two allelic gens, M and N (Springer et al., 1972a). As the genes are co-dominant, the three possible phenotypes MM, MN and NN are found. M and N antigenic activity is associated with glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane (Azuma et al., 1973; Tuech and Morrison, 1974; Tomita and Marchesi, 1975), whereas the closely related Ss antigens are associated with glycophorin B on the same membrane (Dahr et al., 1975a; Tanner et al., 1977; Anstee et al., 1979). An interesting piece of evidence concerning the assignment of MN antigen activity to glycophorin A resulted from the discovery that certain rare individuals with erythrocytes of type En(a-) have a total deficiency of glycophorin A and that this deficiency correlated with a gross reduction in MN antigen activity (Tanner and Anstee, 1976; Dahr et al., 1976a, b; Furthmayr, 1978).

Glycophorin A has been extensively studied and is now known to contain a polypeptide chain composed of 131 amino acids (Tomata et al., 1978) and spanning the membrane with its amino terminus external to the

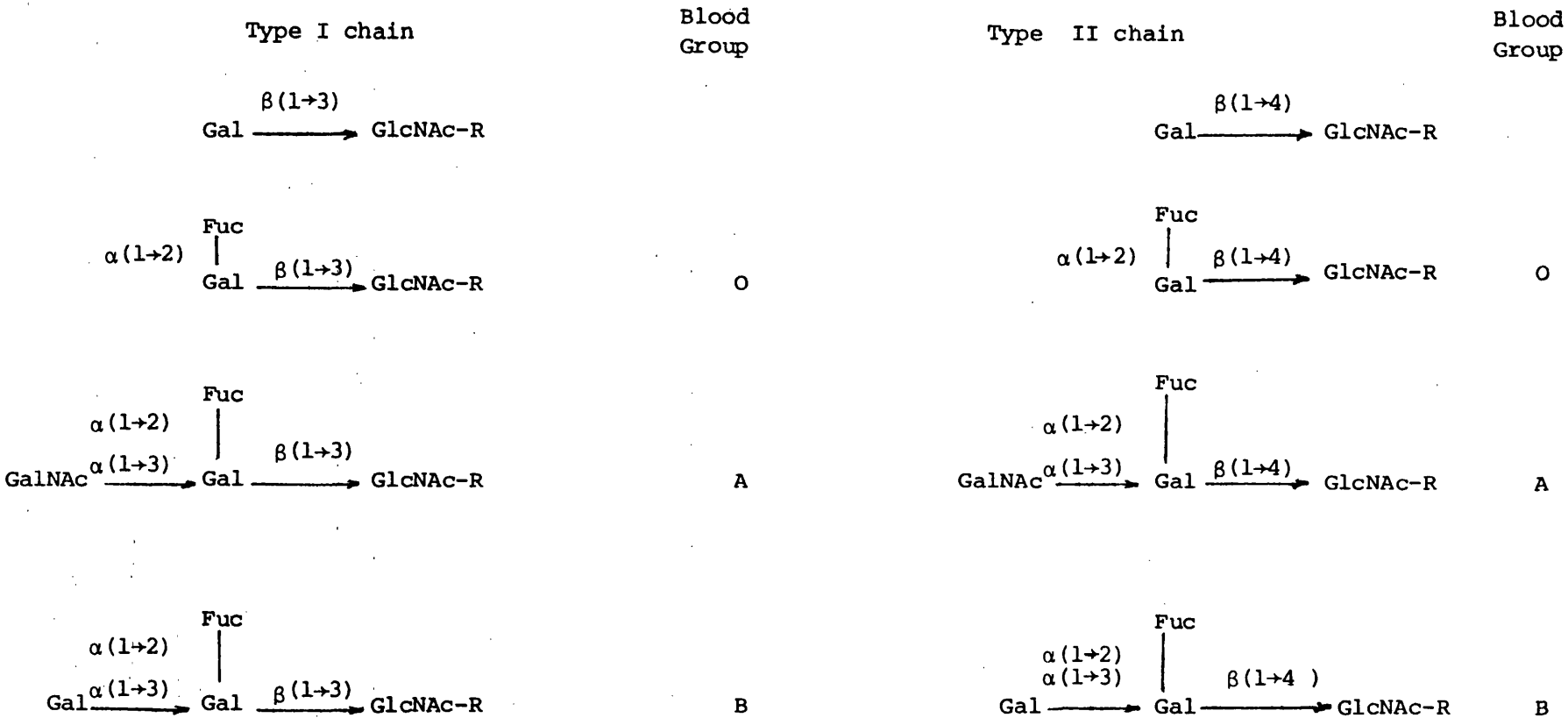


Fig. 14. Oligosaccharide determinants controlling human ABO group antigenicity (After Hughes, 1975).

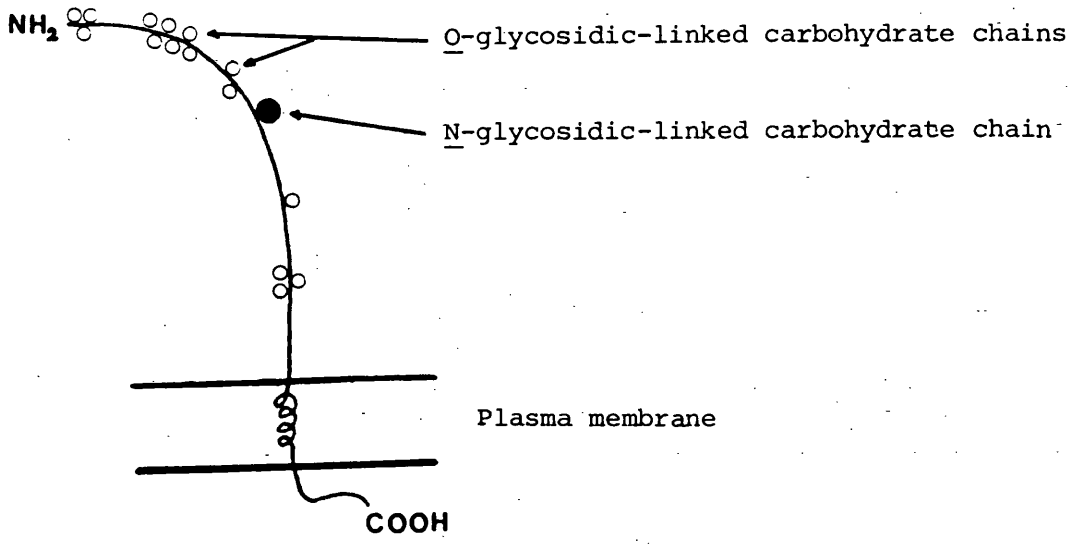


Fig. 15. Schematic diagram of glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane.

cell and its carboxy terminus in the cell cytoplasm (Fig. 15). Glycophorin A consists of approximately 60% carbohydrate, all external to the cell and comprising 15 O-glycosidically linked oligosaccharides and 1 N-glycosidically linked oligosaccharide complex (Fig. 15). Its oligosaccharide portion has been modified by periodate (Lisowska and Duk, 1972; Lisowska and Roelcke, 1973) or alkaline borohydride (Lisowska, 1969; Thomas and Winzler, 1971; Lisowska and Duk, 1975) treatments which resulted in the destruction of MN activity. Such activity is also lost following the removal of sialic acid from intact erythrocytes (Springer and Ansell, 1958), or from glycophorin (Romanowaska, 1969) and its proteolytic fragments (Lisowska and Wasniowska, 1978). These observations suggest that O-linked oligosaccharides which contain sialic acid contribute to the antigenicity of M and N active structures. When antibodies directed against M or N determinants were raised in rabbits, it was noted that anti-N sera

agglutinated MM cells in addition to a much stronger reaction with NN cells. However, monospecific anti-M serum showed no cross-reaction with NN cells (Springer et al., 1972). This observation together with the fact that treatment of MM cells with neuraminidase results in an transient increase in their N-activity (Yokoyama and Trams, 1962) prompted the proposal that the N antigen is a precursor substance which can be sialyated to form M antigen (Springer and Desai, 1975) as shown in Fig. 16. Despite the above observations, however, no difference between oligosaccharides derived from M or from N antigens has been found (Lisowska, 1969; Lisowska et al., 1980) and it is now generally accepted (Dahr et al., 1975b;1977; Furthmayr, 1978), that the genetically determined differences between M and N glycoproteins are located in the polypeptide chains. Covalent modification of the glycophorin polypeptide has been shown to alter the M and N antigenicity detected by both human and rabbit antisera as well as by N-specific lectins (Ebert et al. , 1972; Lisowska and Duk, 1975a,b) and it has been established (Lisowska and Wasniowska, 1978; Blumenfeld and Adamany, 1978) that the nature of the amino acids at positions 1 and 5 (from the amino-terminus) of glycophorin A correlates with the expression of M or N antigen activity on the molecule (Fig. 17).

The role of alkali-labile oligosaccharide complexes in the expression of MN activity can be best explained in terms of their effect in determining the optimal conformation of glycophorin A for such activity. This conformation is probably primarily effected by charge interactions between carbohydrate (sialic acid) carboxyl groups and polypeptide amine residues (Anstee, 1981). Mutual stabilization of peptide and carbohydrate conformations by noncovalent association seems to occur in IgG where an N-glycosidically linked oligosaccharide

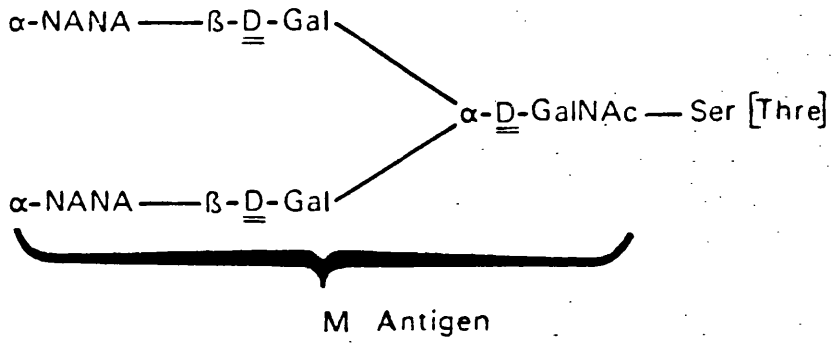


Fig. 16. Structural relationship between M and N antigens as proposed by Springer and Desai (1974).

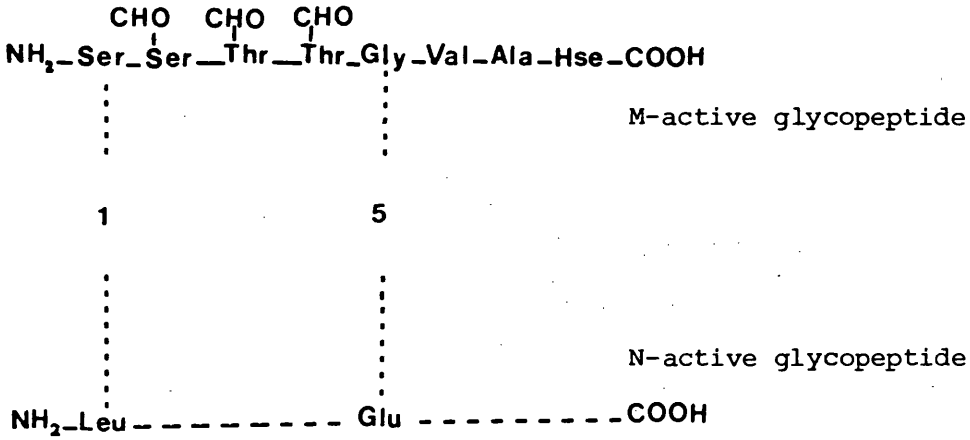


Fig. 17. Amino acid sequence of the smallest peptide fragments that have been shown to retain M or N activity (After Lisowska and Wasniowska, 1978).

exists in an extended sheet-like conformation with many contacts with the peptide sheet (Huber et al., 1976). The view of Springer et al., (1977) that the structural difference between M and N antigens is due to the presence of an M-specific sialyltransferase, absent from individuals with NN cells, has been contested by Sadler et al., (1979) who showed that purified homogeneous sialyltransferases from porcine submaxillary glands are capable of restoring M antigenic activity to neuraminidase-treated M-positive erythrocytes but not to neuraminidase-treated cells that are M-negative prior to desialyation.

The MN system together with its related antigens and glycophorin structure and function have been recently reviewed by Anstee (1981).

(iii) Receptors for bacterial toxins and glycoprotein hormones

In addition to their role as cell surface antigens, membrane-bound glycolipids and glycoproteins have the capacity to act as receptors for a range of extracellular agents such as hormones and bacterial toxins. In this respect, the clearest example is the function of gangliosides as cell plasma membrane receptors for certain toxins and hormones (for review see Fishman and Brady, 1976; Kohn, 1978; Kohn et al., 1978).

Early studies (Holmgren et al., 1973; King and van Heyningen, 1973; Cuatrecasas, 1973) on the interaction of cholera toxin with epithelial membranes showed that the toxin was specific for the oligosaccharide chains of G_{M1} ganglioside (Fig. 18). Subsequently, Holmgren et al. (1974) showed that the sphingosine portion of the molecule was also essential for toxin-binding. Kanfer et al. (1976), on the other hand,

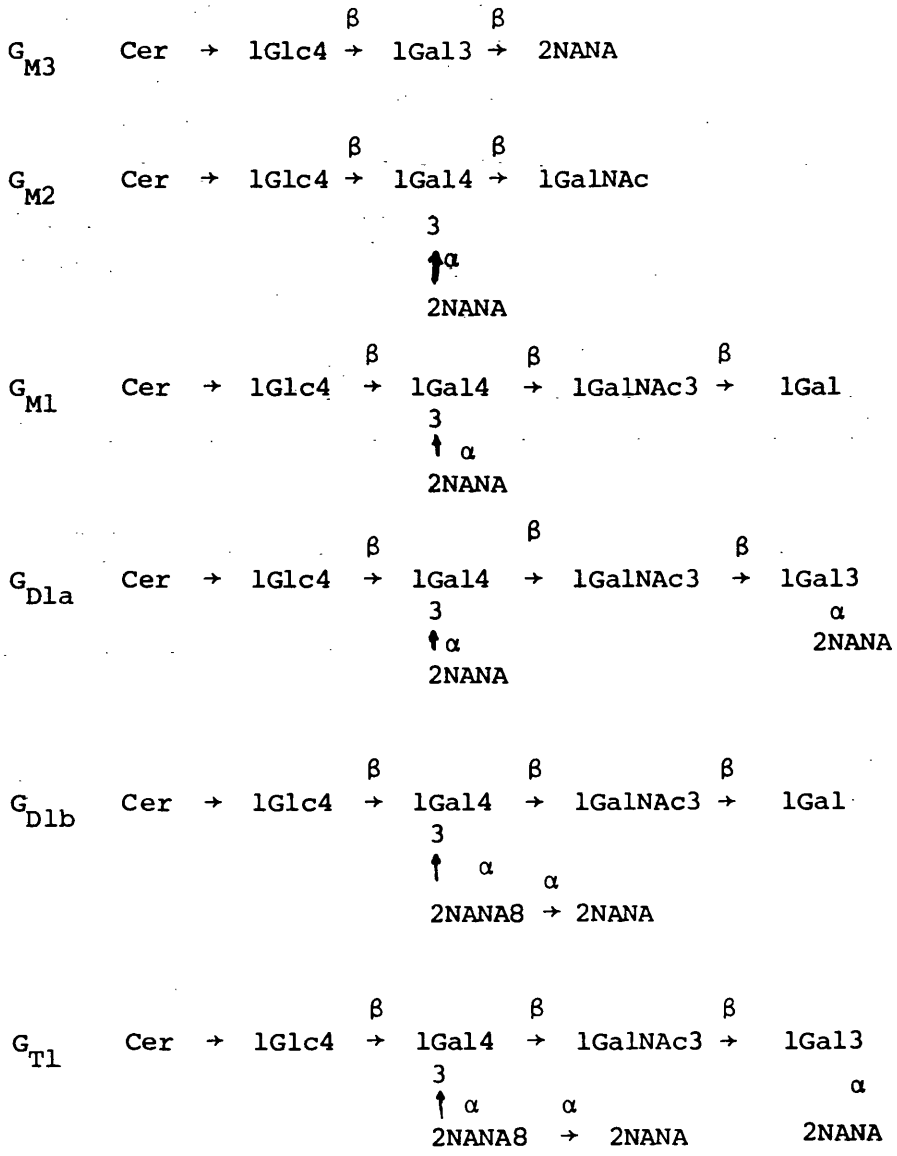


Fig. 18. Structure of the gangliosides

suggested that G_{M1} is not the receptor for cholera toxin in adipocytes as they were found to contain only G_{M3} and G_{M2} (Fig. 18). Furthermore, although exogenously added G_{M1} enhanced the effect of cholera toxin on whole cells, the kinetics of stimulation (lypolysis) did not conform to the activity observed with untreated cells. This conclusion was later questioned by Pacuszka et al. (1978) who showed that G_{M1} was present in adipocytes in low concentrations.

Like cholera toxin, tetanus toxin has been shown (van Heyningen, 1976) to bind to gangliosides such as G_{D1b} and G_{T1} which contain two internal sialic acid residues (Fig. 18), while the haemolytic activity of Staphylococcal alpha toxin has been shown (Kato and Masahara, 1976) to be inhibited by the glucosamine-containing ganglioside sialyl-α2, 3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-ceramide.

Other toxins with similar abilities to bind to gangliosides have been recently reviewed by Kohn (1978).

Although the above findings are of great importance, they do not in themselves reveal the normal biological roles of gangliosides, as cells are not usually exposed to toxins. One possibility is that the toxins are chemical analogues of natural substances such as hormones and interferon and that the true role of relevant gangliosides is to act as membrane receptors for these agents. Indeed, receptors for thyrotropin (Mullin et al., 1976), leuteinizing hormone (Kohn, 1978), human chorionic gonadotropin (Lee et al., 1976) and follicle stimulating hormone have all been shown to be gangliosides. However, recent studies showed that in addition to the ganglioside

receptor, at least another component, a glycoprotein, is involved in the binding of thyrotropin to thyroid plasma membrane (Meldolesi et al., 1977). From these and other results (Kohn, 1978), it is difficult to reach a clear picture of the molecular nature of cell surface receptors for glycoprotein hormones but it is likely that carbohydrate receptors on both glycolipids and glycoproteins are involved in the cell membrane recognition sites. Further information may well depend upon the isolation by affinity procedures of the actual receptor molecules.

It is of interest to mention that investigation of the adherence of several strains of E. coli to human epithelial cells has shown that E. coli adhere by binding specifically and reversibly to D-mannose residues on the target membranes (Ofek et al., 1977, 1978). Similarly, the attachment of E. coli and Salmonella typhi to mouse peritoneal macrophages has also been found to be specifically inhibited by D-mannose, methyl-α-D-mannoside and yeast mannan (Bar-Shavit et al., 1977). Adherence and its relation to pathogenicity will be discussed in Section C.

(iv) Liver receptors for desialyated plasma glycoproteins, erythrocytes and lymphocytes

One of the best known examples of carbohydrate recognition by membrane-bound glycoproteins is the binding by hepatocytes of asialo-glycoproteins. This activity was first discovered by Morell et al. (1968) who showed that desialyated serum glycoproteins were rapidly cleared from the circulation by the liver. Subsequently, Pricer and Ashwell (1971) reported that plasma membranes of rat liver hepatocytes specifically bound asialoglycoproteins through galactose

residues exposed by the removal of sialic acid. Sialic acid itself was also implicated as an essential part of the functional receptor molecules themselves (Ashwell and Morell, 1974). The rabbit hepatic receptor was isolated and shown (Hudgin et al., 1974) to be an integral glycoprotein containing 10% of its dry weight as carbohydrate. Although removal of terminal sialic acid leads to loss of receptor activity, it is likely that this loss results from binding of the receptor's own exposed galactose residues rather than to an essential role of sialic acid in binding (Paulson et al., 1977). Subsequent studies showed that the glycoprotein receptor consists of two subunits (A and B) of molecular weight; 48,000 and 40,000 forming aggregates of molecular weight 500,000 (Kawasaki and Ashwell, 1976a). Pronase treatment of the intact receptor allowed the isolation of two distinct glycopeptide fractions for which the partial structures shown in Fig. 19 were proposed (Kawasaki and Ashwell, 1976b). Similarly, Baenzinger and Maynard (1980); isolated the human hepatic receptor and showed it to be an integral glycoprotein comprising a single subunit (mol. wt. 41,000). The amino acid composition of the human receptor was shown to resemble that of A and B subunits of the rabbit hepatic receptor whereas the carbohydrate composition more closely approximated to that of the A subunit. Inhibition studies showed that the human hepatic receptor displays specificity for both terminal galactose and N-acetylgalactosamine. It is of interest to mention that, while the hepatic binding process in mammals involves the interaction of the receptor with galactose and possibly N-acetylgalactosamine, it involves N-acetylglucosamine in birds and possibly reptiles (Ashwell and Morell, 1977; Weir, 1980).

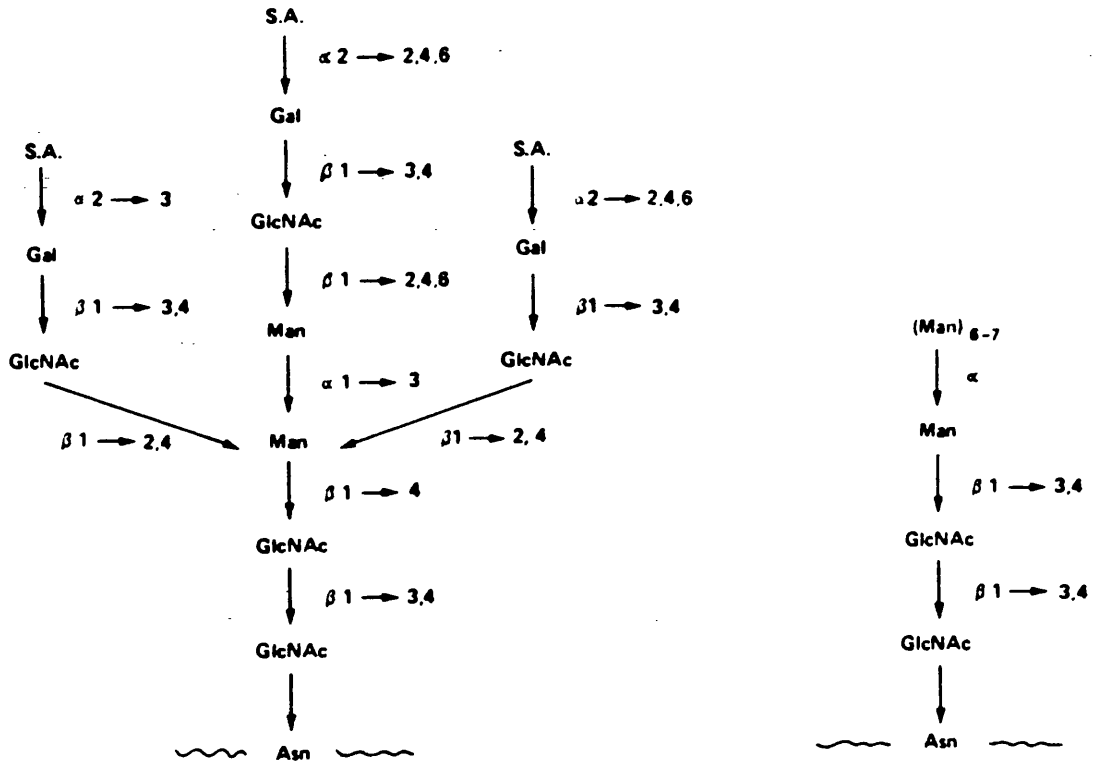


Fig. 19. Partial structures proposed for the oligosaccharide complexes of two glycopeptides from the asialoglycoprotein receptor of mammalian liver plasma membranes (After Kawasaki and Ashwell, 1976b).

As with asialoglycoproteins, neuraminidase-treated erythrocytes are rapidly eliminated from the circulation by the liver (Aminoff et al., 1976, 1977; Kolb et al., 1978). It was suggested that the hepatic cells recognize sialic acid-depleted erythrocytes by interacting with subterminal galactosyl residues that are exposed by the neuraminidase treatment (Kolk et al., 1978). However, there is no clear evidence to suggest that this is the true mechanism of erythrocyte elimination in vivo (Anstee, 1981). Kolb and Kolk-Bachofen (1978) pointed out that asialoerythrocytes apparently bind to Kupffer cells which should remove the red cells by endocytosis whereas glycoproteins could be metabolised exclusively by hepatocytes.

The mammalian hepatic receptors have been classified as mammalian lectins on the basis of their ability to agglutinate red blood cells (Stockert et al., 1974) and to induce mitosis in lymphocytes (Novogrodsky and Ashwell, 1977). Similar lectin activity has been reported to be present in lymphocytes (Kieda et al., 1978), calf heart (Childs and Feizi, 1979) and embryonic chick muscle (Kobiler and Barondes, 1979) extracts. The true biological functions of these surface carbohydrate-receptors remain to be determined.

Lectins, their function and biological activities will be discussed in Section B.

(v) Carbohydrates in Cell-Cell adhesion

Intercellular adhesion is a fundamental biological property of cells in multicellular organisms and it is certainly important in a wide variety of physiological processes including growth, morphological

differentiation and metastasis (Curtis, 1962). Oppenheimer et al. (1969) used trypsin treatment to isolate single cell suspension of mouse teratoma tumours which, when placed in a synthetic tissue culture medium, showed rapid reassociation of individual cells. However, the recovery of adhesiveness required the presence of glutamine which could be specifically replaced by glucosamine or mannosamine. The conclusion drawn from this was that conversion of non-adhesive to adhesive cells correlates with the ability of the cells to synthesize complex carbohydrate. The involvement of cell surface carbohydrate in cellular adhesion has also been studied by treating cells with specific glycosidases; Roth et al. (1971) showed that treatment of dissociated neural retina cells with β-galactosidase alters their adhesive properties. Similarly, the preferential adhesion of dorsal retinal cells to the ventral half of the tectum was prevented by treatment of the retinal cells with β-N-acetylhexosaminidase or by treatment of the tectum with proteases (Marchase, 1977). It was concluded from these experiments that one of the ligands involved in retinal-tectal adhesion is a ganglioside (G_{M2}) with a terminal GalNAc residue, and that the other is a protein molecule which can bind to G_{M2}, possibly a galactosyl transferase. This conclusion was supported by the observation that liposomes containing G_{M2} bound preferentially to dorsal tectal halves (Marchase, 1977). The involvement of cell surface glycosyltransferases in cell-cell adhesion had, in fact, been postulated in a general hypothesis (Roseman, 1970), in which adhesion was suggested to be mediated by enzyme-substrate recognition. However, this hypothesis has not since been satisfactorily supported by experimental data and while it certainly stimulated considerable work is not now generally regarded as a fundamental mechanism of cell-cell adhesion (Culp, 1978). It is quite likely that

the functional role of cell surface enzymes, if any, will be clarified only when they can be purified and when antibodies to them have been prepared. Both the pure enzymes and their antibodies can then be tested as potential inhibitors of adhesion (Culp, 1978).

In view of the presence of lectins in plasma membranes and subcellular membrane fractions, of a range of mammalian species, Ashwell and Morell (1977) suggested that carbohydrate-mediated cellular and intracellular recognition phenomena may be regarded as direct manifestations of native, endogenous mammalian lectins. This suggestion is not dissimilar to the hypothesis of Roseman (1970) but provides for a better appreciation of the properties of mammalian receptors such as the hepatic binding protein which is totally devoid of glycosyl transferase activity (Hudgins and Ashwell, 1974) and which exhibits lectin-like characteristics in promoting erythrocyte agglutination (Stockert et al., 1974) and in stimulating mitogenesis in lymphocytes (Novagrodsky and Ashwell, 1977). More recently,Kobiler and Barondes (1979) isolated a lectin from embryonic chick muscle and suggested that the lectin might play a role in interaction of developing muscle cells with components of the extracellular matrix. Similarly, Childs and Fezi (1979) isolated from calf heart a lectin that is specific for blood group Ii antigens. The fact that they also reported the presence of receptors for this lectin in calf heart extract could suggest that the lectin might play a role in the interaction between calf heart cells. The presence of similar lectins in the human liver and in rat Kupffer cells has been discussed above.

Research over the past few years has indicated the involvement

in cell adhesion of glycoproteins with subunit molecular weight of 200,000 - 250,000. These glycoproteins are now thought to be closely related, and are termed fibronectins (Yamada and Olden, 1978). Fibronectins from various sources probably represent just two specific proteins, cell surface and plasma fibronectins respectively. Cell surface fibronectin [also known as LETS, large external transformation-sensitive, (Hynes and Bye, 1974) or CSP, cell surface protein (Yamada and Weston, 1974)] is a major constituent of the cell surface of many cultured cells, and was discovered when cell surface proteins or carbohydrates were labelled radioisotopically or immunologically. Plasma fibronectins [also known as Cig (Cold insoluble globulin)], on the other hand, circulate in vertebrate blood (Iwanaga et al., 1978) Current information suggests that although the two forms of fibronectin are very similar, they are probably not identical (Yamada and Older, 1978).

The first evidence suggesting that cellular fibronectin may play a role in cell-cell adhesion was the finding that fibronectin purified from cell surfaces behaves like a lectin and readily agglutinates fixed erythrocytes (Yamada et al., 1975; Baum et al., 1977). Purified cell surface fibronectin can also increase aggregation of living dissociated chick embryo and baby hamster kidney (BHK) cells (Yamada et al., 1978).

Serum factors are required for the attachment of certain cells to collagen-coated dishes (Pearlstein, 1976; Hynes, 1978) or for cells to attach and spread on tissue culture dishes (Grinnell, 1975a, b,, 1977). These cell attachment and cell spreading factors are now thought to be

serum fibronectin (Hook et al.,1977). Such serum factors can bind directly to collagen or to tissue culture dishes in the absence of cells (Grinnell et al., 1977)

In spite of our knowledge about structure, location and probable function, the mechanism by which fibronectin interacts with cells and with other adhesive molecules remains unclear.

(vi) Histocompatibility (transplantation) antigens;

A major histocompatibility complex (MHC) has been detected in all mammalian species so far studied (Ting, 1981). The two groups of histocompatibility antigens are the classical major transplantation antigens (HLA-A, B and C in man; H-2K, D and L in the mouse) and the I-region-associated antigens (HLA-DR_w in man and Ia in the mouse) (Owen and Crumpton, 1980). The primary function of the MHC is immune regulation of T-lymphocyte receptor and effecotr functions (Zinkernagel and Doherty, 1979).

The HLA-B and C antigens are among the most extensively characterised plasma-membrane glycoproteins. Their structures appear to be closely related and are viewed as comprising four domains of the external surface of the lipid bilayer (Fig. 20). One of these is β_2-microglobulin (a non-glycosylated protein associated with HLA-A and B antigens) and the other three (α_1, α_2 and α_3) are located in the papain-cleaved fragments of the heavy chain (Fig. 20). A hydrophobic stretch of the heavy chain traverses the lipid bilayer of the plasma membrane (for review see Owen and Crumpton, 1980). It is currently believed that the antigenic specificity of the glycoprotein

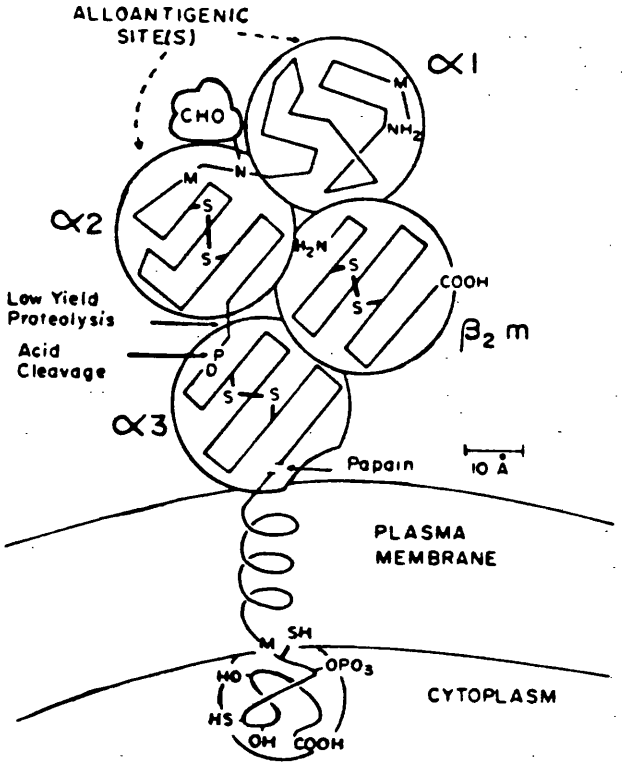


Fig. 20. Schematic representation of an HLA-A or B molecule in the plasma membrane. The molecule is viewed as comprising of four deomains at the external surface of the lipid bilayer. β_2-microglobulin comprises one domain; the α_1, α_2 and α_3 domains are formed by the heavy chain. Digestion of the plasma membrane or detergent-solubilized HLA-A and B antigens with papain produces a water-soluble fragment of apparent molecular weight 34,000 which retains alloantigenicity. (After Own and Michael, 1980).

residues in its amino acid sequence (Orr et al., 1979) rather than in the carbohydrate side chain.

The serological determinant of H-Y (a minor histocompatibility antigen), on the other hand, was shown (Shapiro and Erickson, 1981) to be dependent on a carbohydrate. However it was not established whether the determinant itself is a carbohydrate or is affected by a carbohydrated. The histocompatibility-Y-antigen has been isolated from cell surfaces of vertebrates and invertebrates and is thought to be a male determining substance in mammals because of its almost perfect correlation with maleness among a variety of mammalian species (Pechan et al., 1979; Shalev et al., 1980).

The Milk Fat Globule Membrane

Milk fat exists as minute globules with diameters ranging from 0.1 - 20 μm (Walstra, 1969). The stability of these fat globules is almost entirely dependent on the interfacial layer at the globule surface, which separates the lipid from the aqueous environment of the milk serum.This layer is known as the milk fat globule membrane (MFGM), an understanding of which is clearly relevant to the storage and processing of milk and cream. The MFGM has, however,a more fundamental significance in that it is now generally accepted to be derived direclty from the apical membrane of the secretory cell. As such, the membrane provides a convenient and easily obtainable source of defined mammalian cell membranes which can serve as a model for structural studies of biological membranes in general. In so far as the MFGM represents the mammary epithelial cell membrane in particular, it can serve as a source of mammary or epithelial cell antigens which are of potential medical importance in breast cancer, pathogenic bacterial adhesion and in a range of immunological phenomena. Interest in the membrane has accordingly grown in recent years and the present introduction will attempt to review the current state of knowledge concerning its origin and properties.

(i) Historical Background

After the discovery in 1674 by Van Leeuwen hoek of fat globules in milk, it was Ascherson (1840) who first recorded the presence of an emulsion-stabilizing substance surrounding the globules. He proposed that the 'haptogenic membrane' was formed as a result of 'capillary condensation' and subsequent aggregation of

albumin at the fat globule surface. Babcock (1885) compared the fat globules of milk with artificial emulsions of the oil-in-water type, which were known to be stabilized by an adsorbed phase, possibly a thin film of serum proteins. He later (1889) reported that he had identified small percentages of 'Lacto-fibrin' in milk andt that it funcioned as a true membrane on the globule surface. Early attempts to identify the material coating the surface of fat globules were faced with difficulties arising from the fact that milk contains numerous proteins each of which is capable of stabilizing the fat phase. To overcome this problem Storch (1897) washed bovine cream with water at 35°C to remove these proteins before extracting the fat with alcohol or ether, leaving the membrane proteins. Palmer and Samuelson (1924) were the first to isolate and partially characterise MFGM material. They detected high concentrations of phospholipids in the membrane, establishing for the first time that substances other than proteins were involved. Palmer and Weise later (1933) demonstrated that phospholipids made up 17.5 - 20.4% of the membrane material and showed that lecithin (phosphatatidylcholine), cephalin (phosphatidylethanolamine) and sphingomyelin were present. In addition they prepared an alcohol-insoluble, but ether-soluble fraction which contained neutral lipid composed of high-melting saturated fatty acids. These early studies were comprehensively reviewed by King (1955) who summarized the data, available at that time, concerning the isolation, origin, structure and composition of the MFGM. He proposed a two-layered membrane structure consisting of an inner-layer of high-melting triglycerides bounded by an outer-layer of uncoiled protein attached to the surface of the fat by a layer of phospholipids (Fig. 21).

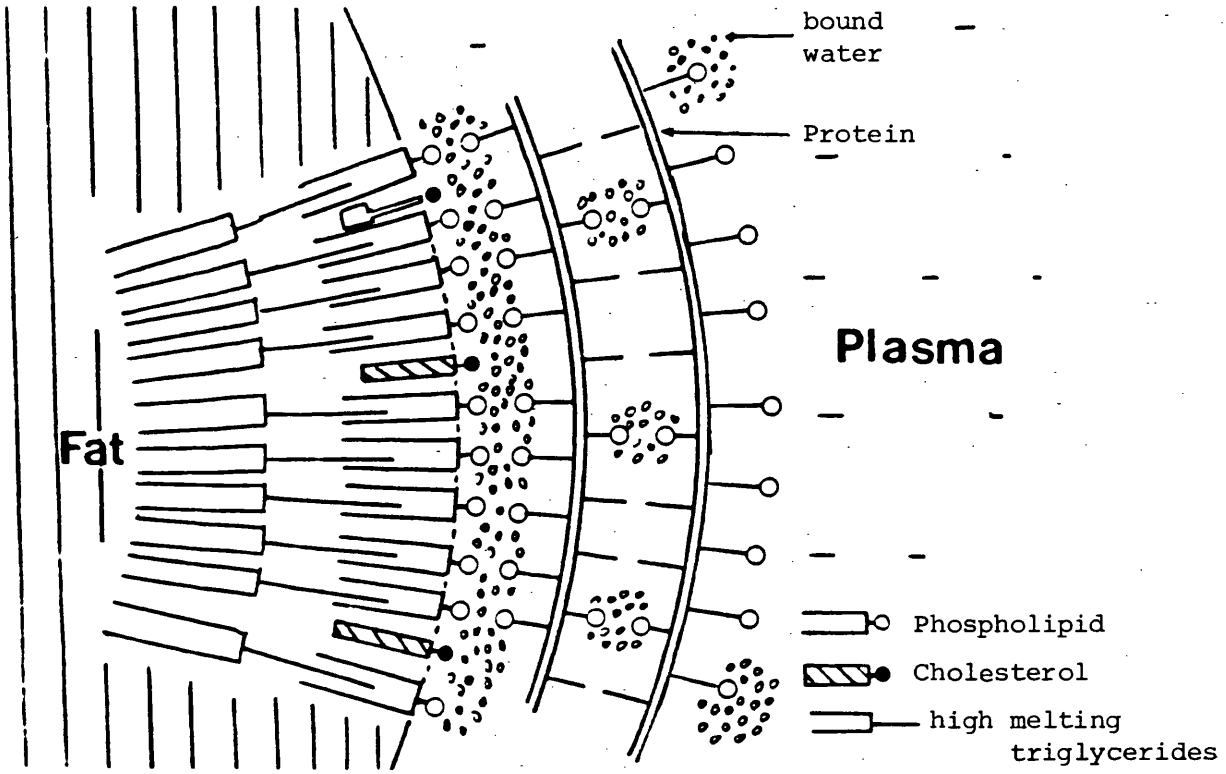


Fig. 21. After King (1955).

(ii) Origin and Structure of the MFGM

As seen from the above discussion, early workers accepted the idea that the interfacial layer at the surface of the fat globule was composed of milk components adsorbed to the fat. Bargman and Knoop (1959), using electron microscopy, presented the first clear evidence concerning the true nature of the membrane. Their electron-micrographs showed the progressive envelopment of the emerging milk fat globule by the apical cell membrane of the secretory cell. Finally the fat globule was seen to be released into the lumen surrounded by an intact membrane. This mechanism of secretion was represented diagrammatically by Bargman and Welsh (1969) (Fig. 22).

As this mechanism of secretion involves the loss of membrane material from the apical regions of the mammary cell, Patton and co-workers (Patton and Fowkes, 1967; Patton and Hood, 1969) introduced a concept of membrane differentiation and flow involving fusion of the Golgi vesicle membrane with the plasmalemma (Fig. 23) which would tend to make good such losses.

One problem with this concept, is that the amount of membrane present in surface-associated vesicles in the lactating cell greatly exceeds that removed by milk fat globule secretion. This problem is discussed by Patton and Jensen (1975). Wooding (1971a) produced electron micrographs which showed that Golgi vesicle membranes were directly involved in the secretion process and he later (1973) demonstrated the possibility of the formation of the MFGM, under certain conditions in goats, from the Golgi vesicle membrane without the participation of the plasma membrane. This observation appeared to be limited to one group of animals but nevertheless emphasizes the important role of the Golgi vesicles in the secretion of the milk fat. Wooding et al. (1970) reported that a small percentage (1 - 5%) of the fat globules in the milk of various species included small crescents of cytoplasm trapped between the fat droplet and the surrounding membrane, but very little cytoplasmic material is carried away by the fat globule, in this way. These crescents sometimes contained mitochondria and other cell organelles, a fact which was subsequently supported by chemical evidence (Swope and Brunner, 1968). Hood and Patton (1973) isolated intracellular milk fat droplets from bovine mammary tissue and found no evidence for the presence of a true membrane surrounding these droplets. However, the droplets appeared

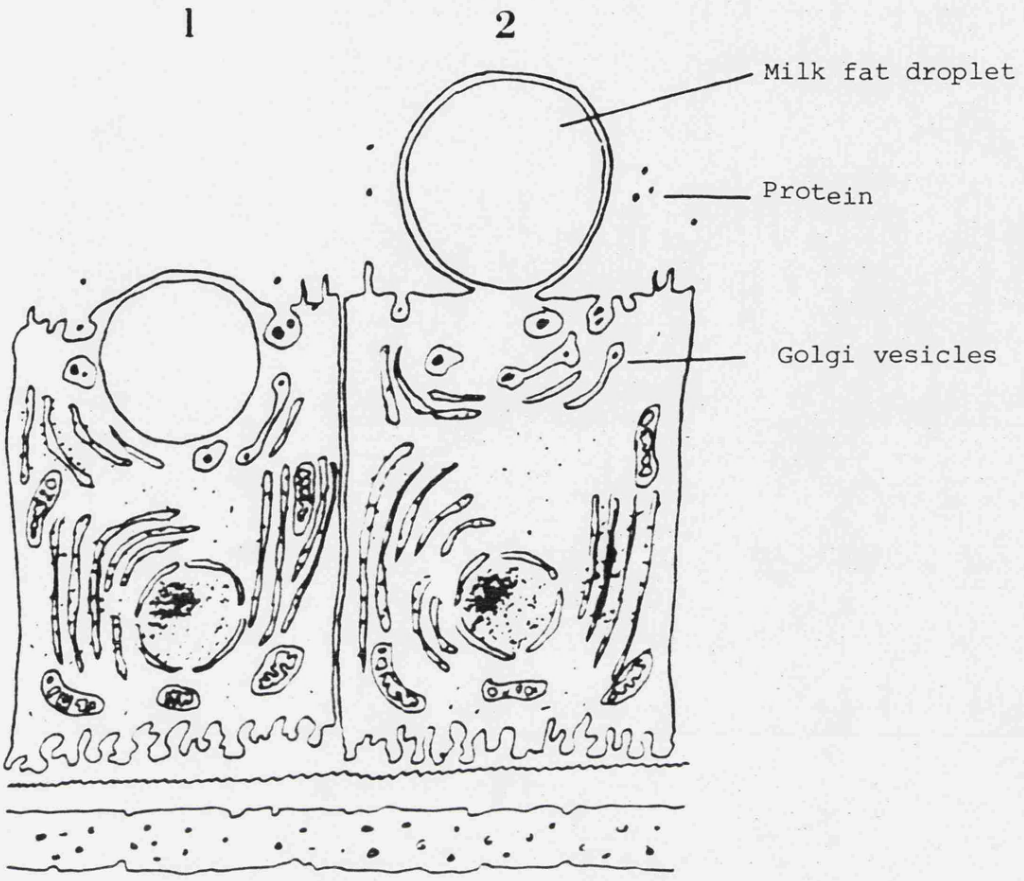


Fig. 22. After Bargmann and Welsch (1969)

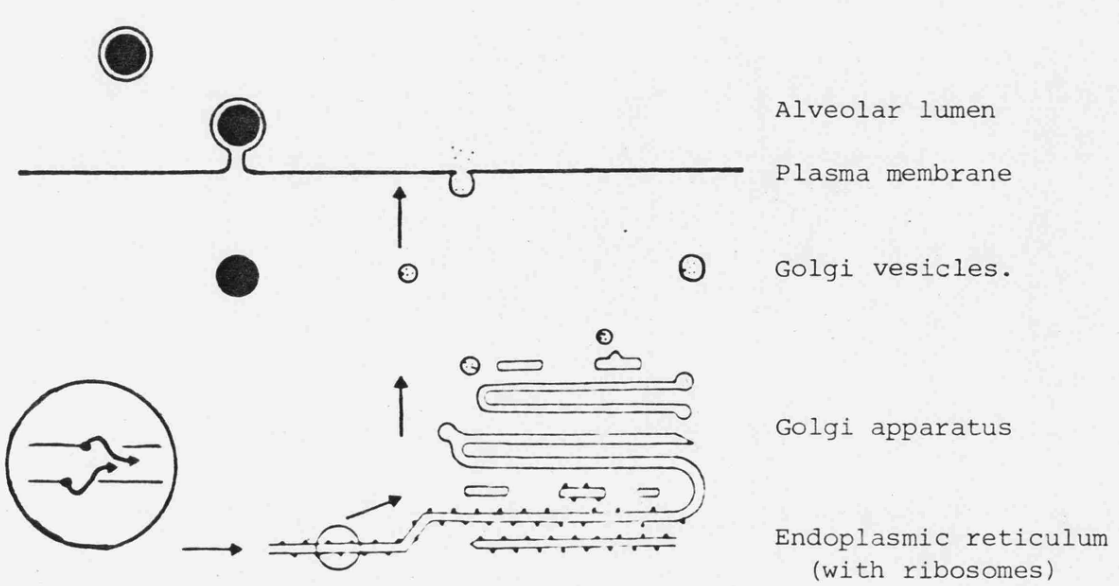


Fig. 23. After Patton and Keenan (1975).

to be enveloped by a discontinuous 'osmiophilic' layer and this was reported to be composed of a phospholipid-cholesterol film containing a small amount of adsorbed protein. It was thought that the phospholipid originated from the endoplasmic reticulum and the proteins were adsorbed from the cytoplasm to stabilize the globule.

Whereas it is generally agreed that, immediately after secretion from the mammary cell, the milk fat globule is enveloped by an intact biological membrane, the subsequent fate of this membrane has been the subject of debate. Electron micrographs suggested that structural changes start during (Farrell and Thompson, 1964; Kurusomi et al., 1968) or directly after (Keenan et al., 1970; Wooding, 1971b, Carroll et al., 1972) secretion. In view of the fact that the MFGM originates from a typical bilayer membrane it might be expected that its juxtaposition with non-polar fat globules would initiate structural rearrangement. Rearrangements of this type have indeed, been demonstrated in the anologous chylomicrons by Zilversmit (1968). While Keenan et al. (1971) explained compositional differences between MFGM and plasma membrane, in these terms, Stewart and Irving (1970), on the other hand, observed that a dense layer (15 - 25 μm) of loosely-adsorbed material is present at the globule surface and suggested that this may be present within the secretory cell prior to its envelopment by the plasma membrane. Such an inner layer of polar material could well obviate the need for rearrangement of the outer MFGM. Even more dramatic changes were suggested by Wooding (1971b) who concluded, from electron microscopic evidence, that the 'primary' MFGM was actually shed from the globule surface leaving a structureless 'secondary' membrane. Chemically there is little support for microscopic

evidence of extensive post-secretory changes in the membrane and both chemical and enzymic contents are generally reported to be unaffected by secretion (Patton and Trams,1971; Baumrucker and Keenan, 1973). Bauer (1972) has examined the effect of preparative procedures on the electron microscopic appearanceof the MFGM, and concluded that conventional methods of fixation may lead to artefactual displacement of the membrane from the milk fat globule. Indeed, Patton (1973) reported that variations in ultrastructure can be produced by different types of fixation whereby it is possible to get double lines, single lines and layered effects on the same globule. The scanning electron micrographs of Horisberger et al. (1977) made use of gold-labelled lectins to show that the greater part of the milk fat globule is covered by MFGM and the present view is that while compositional changes in the membrane can certainly be induced by different types of processing (Mulder and Walstra, 1974) freshly expressed milk fat globules largely retain the unit membrane of the mammary secretory cell.

(iii) Isolation of MFGM

As stated above, Palmer and Samulson (1924) were the first to isolate and partially characterise MFGM material. Isolation of the MFGM is generally considered to be a straightforward procedure in view of the almost complete absence of cytoplasmic contamination. The first step in recovery of the globule membrane is to separate fat globules from milk serum. This can easily be achieved by centrifugation either in a conventional centrifuge or in a commercial cream separator. The separated globules are then washed several times with water or isotonic buffer to remove adsorbed milk serum

components. Membrane material can be removed directly from the washed globules simply by churning, freezing-thawing or sonication all of which disrupt the membrane so allowing the globules to coalesce producing butter. The resulting buttermlik constitutes an aqueous suspension of membranes. Conditions for the washing of milk fat globules must be carefully chosen as the possibility clearly exists of removing loosely-associated membrane constituents, as well as adsorbed milk serum components. These problems have been discussed by Brunner (1974) and Mulder and Walstra (1974). Swope and Brunner (1968) showed that three washes with three volumes of deionized water reduced the concentration of serum proteins to acceptable levels. Patton and Keenan (1975) have reported that better eventual yields of membrane are obtained by the use of isotonic wash solutions (sucrose or NaCl) rather than with water or dilute buffer. They attributed this to greater stability of the globules and consequently to less separation of membrane fragments during washing. Anderson et al. (1972) demonstrated that cooling the milk before membrane isolation resulted in a lower recovery of membrane material. Data presented in table (2) clearly show the loss of membrane material as a result of globule washing.

The relative merits of the various disruption procedures have also been studied. Kobylka and Carraway (1972) investigated several procedure in order to optimize the preparation of bovine MFGM. They showed that freeze-thawing in buffered sucrose containing magnesium ions was superior to the homogenization methods investigated. Martel et al. (1973) on the other hand, compared churning and freeze-thawing procedures for preparation of human MFGM and reported that churning gave the higher yields and a superior fraction, based on morphological

Sample	Content in cream (g/100 ml)	
	phospholipids	protein
Original cream	0.45	11.40
Washed once	0.34	1.66
Washed twice	0.24	0.73
Washed thrice	0.22	0.61
Washed 4 times	0.19	0.59
Original cream	0.40	
Washed 4 times	0.35	0.69
Washed 8 times	0.28	0.61
Washed 12 times	0.18	0.54
Washed 24 times	0.13	0.46

Table 2. Loss of materials from fat globules in cream by washing (After Mulder and Walstra, 1974).

and enzymic comparisons.

Patton and Keenan (1975) suggested that for certain studies of membrane constituents, (e.g. enzyme activities, electrophoretic mobility, labelling of external membrane constituents) washed globules can be used as such. Membrane proteins have been obtained directly from intact fat globules by sodium dodecyl sulfate (SDS) treatment (Kobylka and Carraway, 1972; Anderson et al., 1972; Mather and keenan, 1975) and phospholipids have been similarly extracted using organic solvents (Huang and Kukis, 1967; Patton and Keenan, 1971). Moreover washed intact milk fat globules are an ideal starting point for the production of glycopeptide fractions which may be cleaved from the membrane outer surface by proteolytic enzymes (Harrison et al., 1975).

(iv) Composition of MFGM

Almost all data available on the composition of MFGM concern the bovine membrane. Proteins and lipids together account for over 90% of the dry weight of the membrane material (Swope and Brunner, 1970; Thompson et al., 1961) but the relative proportions of these two constituents vary greatly according to many factors including season, diet and stage of lactation in the animal. (Patton and Keenan, 1975). The method of membrane isolation is also relevant in this context (Anderson et al., 1972; Chandan et al., 1971; Walstra, 1972; Anderson and Cawston, 1975) as it involves the separation of membrane material from a fat droplet containing over 95% triglycerides. The gross composition of the membrane according to Patton and Keenan (1975) is given in Table (3).

Constituent	Amount
Proteins	25 to 60% of dry wt.
Total lipids	0.5 to 1.1 mg/mg protein
Neutral lipids	56 to 80% of total lipids
Hydrocarbons	1.2% of total lipids
Sterols	0.2 to 5.2% of total lipids
Sterol esters	0.1 to 0.8% of total lipids
Glycerides	53 to 74% of total lipids
Free fatty acids	0.6 to 6.3% of total lipids
Cerebrosides	3.5 nmol/mg protein
Gangliosides	6 nmol/mg protein
Sialic acids	63 nmol/mg protein
Hexoses	0.6 μmol/mg protein
Hexosamines	0.3 μmol/mg protein

Table 3. Gross composition of bovine milk fat globule membranes (After Patton and Keenan, 1975).

I. Lipids

The lipid fraction of the MFGM was first characterized in detail by Thompson et al. (1961) who demonstrated that the membrane contains phospholipids, triglycerides including many with high melting point (>50°C), mono and diglycerides, free fatty acids, cholesterol esters, carotene and squalene-like substances. Distribution of individual phospholipids were shown to be similar in MFGM and in plasma membranes of the secretory cells (Table 4).

Phospholipid	% of total lipid phosphorus	
	PM	MFGM
Sphingomyelin	23.5	21.9
Phosphatidylcholine	32.9	36.2
Phosphatidylserine	4.4	4.1
Phosphatidylinositol	11.6	10.7
Phosphatidylethanolamine	25.3	27.5
Cardiolipin	0.4	-
Lyso derivatives	1.8	2.3

Table 4. Phospholipid distribution in MFGM and bovine mammary epithelial membrane (Patton and Keenan, 1975).

Triglyceride may account for more than 60% of the neutral lipid of MFGM compared with a corresponding figure of 24% in the plasma membrane of the mammary cell (Keenan and Huang, 1972) while a comparison of the triglyceride fatty acid composition of plasma membrane (Keenan and Huang, 1972) and MFGM shows that, in general, plasma membrane has a much lower content of 16:0 and

18:0 fatty acids than does MFGM. These observations indicate that it is most unlikely that all the triglycerides in MFGM could have arisen from plasma membrane triglyceride and it has been concluded (Walstra, 1974) that much MFGM triglyceride represents fat globule-derived contaminant attached to the membrane during preparation. That this triglyceride is associated with the inner face of the MFGM is supported by the microelectrophoretic studies of Newman and Harrison (1973) which indicated that the outer surface of intact bovine milk fat globules is predominantly ionogenic and contains little neutral lipid. Indeed microscopy shows the presence of a layer of material associated with one face of isolated MFGM and this has been identified as high melting point triglyceride derived from the fat globule itself (Bauer, 1972; Keenan et al., 1970, 1971). A different point of view has been expressed by Wooding (1972) and Martel et al. (1973) who regard this attached layer as representing one or more protein fractions.

Keenan (1974) was able to distinguish six different gangliosides in the bovine MFGM and he reported that 90% of total milk gangliosides are present in the MFGM and represent approximately 8% of the total sialic acid. Detailed analysis of the lipid composition from human and bovine milk was published by Bracco et al., (1972).

II. Proteins

Early studies established that MFGM proteins are different from those of milk as a whole. Membrane preparations have been extracted by various procedures and both soluble and insoluble fractions have been investigated with respect to compositional, immunological, electrophoretic and sedimentation characteristics.

These studies have been extensively reviewed by Brunner (1974).

Using polyacrylamide gel electrophoresis (PAGE), Keenan et al. (1970) demonstrated the presence of nine polypeptides in bovine MFGM and showed that these are similar to those isolated from bovine mammary cell membrane. It has since been found that more than 98% of the MFGM proteins can be solubilized by treatment with SDS and 2-mercaptoethanol (Mather and Kennan, 1975) and electrophoresis in SDS-containing polyacrylamide gels has demonstrated the presence of 5 - 7 major bands in the majority of investigations of bovine MFGM (Anderson et al., 1974 ; 1972; Anderson, 1974; Kobylka and Carraway, 1972; 1973; Mather and Keenan, 1975). It is difficult to compare the results from the different studies, as differing concentrations of acrylamide and acrylamide/bis-acrylamide ratios have been used, and whereas some authors have disregarded minor bands, others have included all bands whatever their concentration. Different methods of preparation and extraction give rise to varying relative preparations of individual MFGM proteins, but the same polypeptides appear to be present in all preparations (Kobylka and Carraway, 1972; Anderson et al., 1974; Anderson and Brooker, 1975). Coomassie-Blue stained electrophoretic patterns of human and bovine MFGM proteins were shown to be somewhat similar (Martel et al., 1973; Mather and Keenan, 1975).

Glycoprotein components of the MFGM have been detected by periodate-Schiff (PAS) staining of polyacrylamide gels. Six glycoproteins have been reported to be present in the bovine MFGM (Kobylka and Carraway, 1972; Anderson et al., 1974 , 1975) whereas only five glycoproteins were observed by Kitchen (1974) and

Mather and Keenan (1975). Anderson et al. (1974) examined the behaviour of bovine MFGM proteins and glycoproteins in several concentrations of acrylamide and calculated the free electrophoretic mobility and retardation coefficient for each protein. From these calculations three of the glycoproteins appeared to be identical to three of the protein bands, indicated by Coomassie-blue staining. This conclusion has since been supported by Mather and Keenan (1975). Combinations of centrifugation and extraction techniques have resulted in the separation of mixtures containing up to seven PAS-staining components (Anderson and Cawston, 1975; Kanno et al., 1975; Shimizu et al., 1976; Murray et al., 1979). Keenan et al. (1977) have used lithium diiodosalicylate to extract a high molecular weight glycoprotein fraction from bovine MFGM. This fraction had three electrophoretically distinguishable glycosylated proteins but was free of nonglycosylated proteins. The isolated final fraction was shown to be water-soluble and antigenic when injected into rabbits. Nielsen and Bjerrum (1977), have established crossed immunoelectrophoretic patterns showing a number of precipitin arcs when detergent-solubilized bovine MFGM was electrphoresed into a gel containing anti-bovine MFGM antisera. The introduction of intermediate concanavalin A gels demonstrated that all the antigenic MFGM components were glycoproteins. Snow et al. (1977) isolated a water-soluble glycoprotein from bovine MFGM. This glycoprotein was purified to approximately 95% purity by hydroxyapatite chromatography followed by gel filtration. Chemical analysis indicated that it contains 50% by weight carbohydrate, 30.5% of which is sialic acid. The other major monosaccharides are N-acetylgalactosamine, N-acetylglucosamine, galactose, mannose and fucose. The major amino acids are leucine, glutamic acid and glycine. Murray et al. (1979) have studied the ability of glycoproteins of

bovine and human MFGM to bind to different lectins after electrophoretic separation. Seven lectin receptor glycoproteins were detected in bovine and five in human MFGM. The abilities of these glycoproteins to interact with certain lectins were also reported to be different. They also separated two major nonionic-detergent-insoluble glycoproteins from bovine and human MFGM. Detergent-insoluble polypeptides with similar or identical electrophoretic mobilities were also reported to be present in the milk fat globule membranes from four other species (rat, sheep, pig and goat). Tryptic peptide mapping revealed these polypeptides to be nonidentical between species.

A different approach to the study of MFGM surface components has involved the treatment of intact milk fat globules with proteolytic enzymes. Treatment of bovine milk fat globules with pronase resulted in the release from the membrane surface of sialoglycopeptides containing, in addition to sialic acid, N-acetylgalactosamine, N-acetylglucosamine, galactose, mannose and fucose (Higginbotham and Harrison, 1972; Harrison et al, 1975; Harrison, 1977; Farrar and Harrison, 1978). Newman et al. (1976a) reported that alkaline borohydride treatment of a crude glycoprotein preparation from bovine MFGM gave a fraction from gel filtration that contained the reduced disaccharide β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosaminitol together with two molecules of sialic acid. Periodate-oxidation and alkaline-degradation studies on the glycoprotein mixture suggested that the major alkali-labile oligosaccharide component was a tetrasaccharide containing β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosamine substituted by sialic acid at position C-3 of the galactose and C-6 of the N-acetyl-D-galactosamine

residue (Fig. 24). Later, Farrar and Harrison (1978) isolated this tetrasaccharide together with the trisaccharides II and III (Fig. 24) which were fully characterised by methylation analysis coupled with periodate-oxidation and gas liquid chromatography. The presence of tetrasaccharide I (Fig. 24) on human MFGM also has been suggested from studies of Newman et al., (1977).

The exposure of specific carbohydrate residues on the outer surface of intact milk fat globules has been little investigated. Microelectrophoretic studies of Newman and Harrison (1973) demonstrated the exposure of sialic acid on the surface of intact bovine milk fat globules, but this technique did not allow the detection of other sugars. Horisberger et al. (1977) in an interesting electron microscopic study have shown the ability of certain gold-labelled lectins to bind in a specific manner to the surface of human and bovine milk fat globules. In view of the possible involvement of sugar residues in a range of cell surface interactions the nature of the exposure of such residues on the outer surface of biological membranes is of particular interest and the MFGM presents a convenient model system for the study of such exposure. Studies of this type accordingly constitute a part of the experimental work described in the present thesis.

III. Enzymes of the MFGM:

Many enzymes have been identified in cream and in isolated MFGM. The amount of each enzyme present is probably influenced by a number of factors including, method of preparation (Mulder and Walstra, 1974; Powell et al., 1977), stage of lactatation (Anderson and Cheeseman, 1975), species (Anderson and Cawston, 1975) and pathological

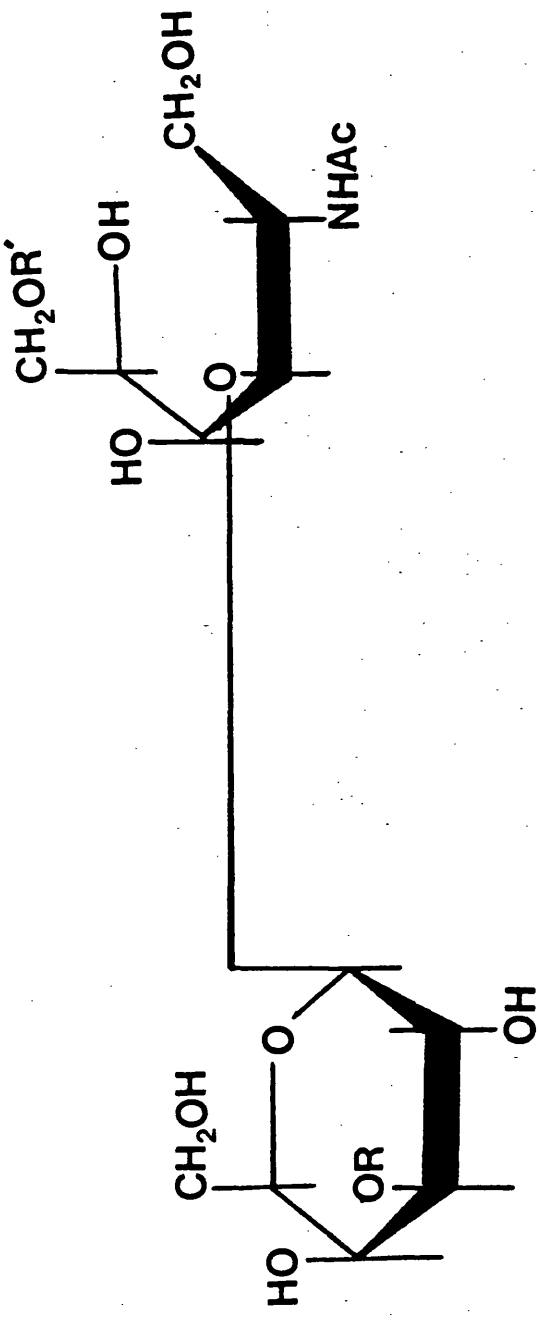


Fig. 24. Structures of alkali labile oligosaccharides isolated from MFGM-derived sialoglycopeptide. (a) Tetrasaccharide I: R=R'=α-N-acetylneuraminyl.

(b) Trisaccharide II: R = α-N-acetylneuraminyl, R'=H. (c) Trisaccharide III; R = H, R' = α-N-acetylneuraminyl (After Farrar and Harrison, 1978).

state (Anderson et al., 1974a, 1975; Anderson and Cheeseman, 1975; Kitchen et al., 1970). Plasma membrane marker enzymes have been detected with high specific activity in bovine MFGM preparations (Solym and Trams, 1973; de Pierre and Karnovsky, 1973; Morré et al., 1974; Trams and Lauter, 1974). Mitochondria (Morton, 1954; Baile and Morton, 1958; Dowben et al., 1967) and endoplasmic reticulum markers (Kitchen, 1974; Dowben et al., 1967; Keenan and Huang, 1972; Plantz et al., 1973) are absent from or present in very low specific activities in bovine MFGM. Results concerning the presence of Golgi marker enzymes in MFGM are less clear. As previously mentioned, Wooding (1971a,1973) reported electron microscopic evidence which implicated Golgi vesicle membrane in the mechanism of milk fat secretion. However, a number of reports (Keenan et al., 1970; Keenan and Huang, 1972; Kennan, 1974) documented the absence of Golgi enzymes in MFGM and although Martel et al. (1972) reported the presence of glucose-6-phosphatase and lactose synthetase (Golgi markers) in human MFGM preparations, they explained the presence of these enzymes as arising from contamination by cytoplasmic components. Powell et al. (1977) suggested that the absence of Golgi markers in isolated bovine MFGM preparations results from their deactivation by proteolytic enzymes at the relatively high temperatures used in the isolation and purification. Galactosyl transferase was in fact found in MFGM and was shown to be similar to that extracted from lactating mammary cell Golgi membrane, and to have a higher molecular weight than the soluble galactose transferase of milk serum.

Dowben et al. (1967) reported the presence of the plasma membrane enzymes acid and alkaline phosphatase and Na^+ and K^+-activated ATPases

in bovine MFGM. Patton and Trams (1971) and Huang and Keenan (1972) have demonstrated that 5'-nucleotidase and Mg^{++}-activated ATPase were present in the MFGM but they were unable to determine the presence of Na^{+}, K^{+} ATPase which has, in fact, been reported to be present in the basal but not the apical plasma membrane of the secretory cell. The presence of Mg^{++}-activated ATPase in bovine MFGM was later confirmed by the immunological studies of Nielson and Bjerrum (1977). Like alkaline phosphatases from Escherichia coli and other mammalian sources, MFGM alkaline phosphatase is usually stable in the presence of SDS (Mather and Keenan, 1974). This property allows detection of alkaline phosphatase in SDS-polyacrylamide gels. Table (5) summarizes the enzymes detected in the MFGM by Dowben et al. (1967).

Enzyme	Origin	Presence in the membrane
Lactate dehydrogenase	cytoplasm	-
Xanthine oxidase	microsomes	+
Succinate dehydrogenase	mitochondria	+
NADH:cytochrome c reductase	microsomes	(little)
Cytochrome oxidase	Mitochondria	-
Catalase	cytoplasm, leucocytes	+
Aspartate aminotransferase	cytoplasm	-
Ribonuclease	lysosomes	-
Lipase	?	-
Acetylcholinesterase	microscomes	+
Alkaline phosphatase	microsomes	+
Acid phosphatase	lysosomes	little
Glucose-6-phosphatase	microsomes ?	+
phosphodiesterase	microsomes	+
Mg^{2+} activated ATPase	microsomes	+
$(Na^{+}\ K^{+}\ Mg^{2+})$activated ATPase	microsomes	+
Aldolase	cytoplasm	little

Table 5. Milk enzymes and the milk fat globule membrane. 'Origin' refers to the probable site of the enzyme before secretion. (After Dowben et al., 1967).

Section A

Isolation and characterisation of sialoglycopeptides from bovine MFGM.

Materials and Methods

I. Materials:

D-galactose, D-mannose, D-glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetylneuraminic acid (sialic acid), L-fucose, L-cysteinehydrochloride and thiobarbituric acid were purchased from Sigma (London) Chemical Co., London, S.W.6, U.K. Pronase (B grade, Streptomyces griseus proteinase) was from Calbiochem Ltd., London, W.1. Sephadex and Sepharose gels were from Pharmacia (G.B.) Ltd., London, W.5. Complete and incomplete Freund's adjuvant were from Miles Laboratories, U.S.A.

The gas chromatographic columns and packings were from Phase Separations Ltd., Queensferry,Flintshire, U.K. Mercaptoethanesulphonic acid and microflux vials were from Pierce Chemical Co., Rockford, Illinois, U.S.A. All other reagents and solvents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were AnalaR grade whenever possible.

SDS markers (IgM μ-chain, IgG γ-chain, IgG K-chain and ovalbumin) were donated by Mr. A.M. Jehanli, University of Bath.

II. Methods

Isolation of Milk Fat Globules:

Fresh cows milk, approximately 24 l, from a Friesian herd in mid-lactation was pooled and maintained within 3 - 4 ^{o}C of the milking temperature in an insulated container. The milk was warmed to 40^{o}C, as soon as possible, and separated in an Alfa-Laval bench cream separator, calibrated to give approximately 40% cream.

The isolated cream (1 vol) was suspended in double distilled water (3 vol) and reseparated. The suspension and separation procedure was repeated three times, after which the cream was suspended in 50 mM Tris-hydrochloride buffer (2 vol), pH 7.8, containing 4 mM $CaCl_2$.

Gel filtration and Ion-exchange Chromatography:

All gel filtration and ion-exchange chromatography procedures were carried out at room temperature. Sephadex gels were swollen by equilibration with water, except for DEAE-Sephadex A-25 which was swollen with 0.65 M pyridine acetate buffer, pH 5. When not in use, columns were stored in the presence of 0.02% (v/v) hibitane (Chlorhexidine) to prevent bacterial proliferation.

Flow rates during column elution were maintained with LKB Varioperspex pump and fractions were automatically collected on an LKB Redirak 2112 fraction collector with an electronic timer. Column eluates were monitored continuously (at 280 or 240 nm) with a Cecil 272 spectrophotometer coupled to a Bryans 28,000 chart recorder.

Protein Determinations:

Total protein in aqueous column eluates was determined colorimetrically by the method of Lowry et al. (1951). Protein in the presence of Triton X100 was estimated by the method of Bonsall and Hurst (1971).

Hexose and Hexosamine determinations:

Total hexose was determined in samples and column fractions colorimetrically by a modification of the cysteine/sulphuric acid assay (Dische and Danilchenko, 1967), using a calibration curve based on D-galactose. Individual sugars, including aminosugars, were determined in the purified sialoglycopeptides and oligosaccharides as their alditol acetate derivatives by gas liquid chromatography (see gas liquid chromatography). Quantitative determinations were done by using alditol acetate derivatives comparing detector responses for individual sugars with those of identically-treated standards.

Sialic acid determinations:

Sialic acid in column eluates was determined by hydrolysing an aliquot (0.1 ml) with 0.2 N-H_2SO_4 (0.1 ml) at 80°C for 1 h, followed by assay of the free sialic acid by using thiobarbituric acid as described by Warren (1959) and modified by Aminoff (1961). In the case of column eluates containing pyridine acetate buffer, aliquots (0.1 ml) were first freeze-dried to remove the volatile buffer and then redissolved in water (0.1 ml) before assay as above.

Gas liquid chromatography

As mentioned above, quantitative estimation of individual sugars in both oligosaccharides and sialoglycopeptides was carried out by analysis of their alditol acetate derivatives by gas liquid chromatography(g.l.c.).This involves three steps, namely acid hydrolysis to release the bound monosaccharides, reduction to the alditol form by treatment with alkaline sodium borohydride and finally acetylation by treatment with acetic anhydride.

Aliquots (0.1 ml) containing purified oligosaccharides or sialoglycopeptides (100 - 200 µg) in 1 ml microflux vials were dried down at 50°C in a gentle stream of nitrogen. 2N-Trifluoroacetic acid (TFA) (50 µl) containing perseitol (10 - 20 µg), as an internal standard, was added to each sample and the vials were sealed and incubated at 121°C for 1 h after which they were dried as described above. 3M-NH_4OH (100 µl) was added to ensure alkaline conditions and the reduction of the released monosaccharides was then carried out by addition of freshly-made sodium borohydride (25 µl) in 3 M-NH_4OH (20 mg/ml). Vials were sealed and incubated for 1 h at room temperature. Excess borohydride was neutralised by addition of glacial acetic acid (1 - 3 drops) followed by addition of dry methanol (100 µl) and drying as described above. The addition of methanol (100 µl) was repeated four times the first of which was carried out by using methanol-water (1:1). Acetylation was carried out by addition of dry acetic anhydride (50 µl) and incubation at 121°C for 1 h. The samples were dried and the residue was dissolved in dichloromethane (50 - 100 µl).

Gas chromatography was performed by injection of samples (0.5 - 1.0 µl) of the dichloromethane solution directly into columns (2 m x 0.25 cm) of coiled glass containing either 3% OV-17 or OV-225 on High performance Gas Chrom Q in a Pye Unicam Series 104 (OV-17) or a Perkin-Elmer, Sigma 3 (OV-225) gas chromatograph equipped with a dual flame ionization detector. Chromatography was carried out isothermally at 205°C (OV-17) or at 200°C (OV-225) with carrier gas flow rates of 45 ml/h.

Standards were prepared by derivatising aliquots (50 µl) of equimolar mixtures of standard hexoses (galactose, glucose, mannose and fucose), hexosamines (N-acetylglucosamine and N-acetylgalactosamine) and internal standard (Perseitol) under the same conditions, described for the samples. Quantitation of individual sugars in the purified sialoglycopeptides and oligosaccharides was achieved by reference to the internal standard using the following formula:

$$\text{Weight of monosaccharide} = \frac{\text{Peak area of monosaccharide}}{\text{Peak area of Perseitol}} \text{ xKFxwt.Perseitol}$$

$$\text{where} \quad KF = \frac{\text{peak area of perseitol}}{\text{peak area of equal wet. of monosaccharide}}$$

Individual sugars were identified by comparison of their retention times with those of the authentic standards.

Amino acid analysis:

Sialoglycopeptide samples (160 µg) in phosphate buffered saline (160 µl), pH 7.3, were dried down under vacuum. The residue from each sample was then hydrolysed in a sealed tube under N_2 at 110°C for 22h with 3N-mercaptoethanesulphonic acid (200 µl). The acid was then neutralised by addition of 1N-NaOH (400 µl) before being injected into the amino acid analyser. The amino acids of the samples were identified by reference to standard amino acids under the same conditions. Samples (200 µl) were injected directly into Chromaspek amino acid analyser (Rank Hilger).

Preparation of Sialoglycopeptides for immunisation

Sialoglycopeptides prepared in phosphate buffered saline, pH 7.3, (2 mg/ml) were diluted by addition of equal volumes of Freund's

complete (first injection) or incomplete (subsequent injections) adjuvant. The emulsion (1.5 ml) was then injected into full-grown New Zealand rabbits at four separate intramuscular sites. Animals were injected at four-weekly intervals, and blood was taken for estimation of antibodies seven days after each injection. Antisera raised in sheep and rabbits against the whole solubilised bovine MFGM were generous gifts from Dr. G. H.Farrar, University of Bath.

Collection of antisera

Blood (10 ml) was taken from peripheral ear veins and allowed to clot for 1 h at 37°C. The clotted blood was then left overnight at 4°C to shrink the clot. Serum was separated and centrifuged (7,000 x g, 15 min) to sediment cells. Aliquots (0.5 ml) of the serum were stored at -20°C until required.

Milk fat globule agglutination assay

Washed bovine milk fat globules suspension (20 µl) was incubated with a serial dilution of antisera (20 µl) and PBS buffer, pH 7.3, (20 µl) for 1 h at room temperature in a humid environment after which the globules suspension was examined at x100 magnification using a light microscope. Control experiments were carried out by using PBS buffer, pH 7.3, in place of antisera.

Inhibition assays were performed by preincubating four agglutination doses of the antisera (20 µl) for 20 - 30 min with inhibitor-containing buffer (20 µl) at room temperature in a humid environment before addition of the globule suspension (20 µl). The mixture was allowed to stand under the same conditions for 1h before being examined, at X100 magnification, by a light microscope.

Agglutination titres were expressed as the reciprocal of the minimum dilution of the antisera giving less than 30% agglutination. Inhibition titres were expressed as the minimum concentration of the inhibitor, in the final volume, required to inhibit four agglutination doses of the antisera. The detailed experimental procedure concerning the preparation of milk fat globules for agglutination assays will be given in Section B.

Immunochemical gel preparation techniques

Gels for double immunodiffusion studies were prepared as follows: 1% (w/v) Agarose (17 ml) together with half its volume of phosphate buffered saline, pH 7,3 containing 0.02% NaN_3 and 1.5% polyethylene glycol with or without 1% Triton X100 was poured onto a glass plate (85 x 95 x 1 mm) and allowed to stand at 4 °C in a humid environment.

Gels for immunoelectrophoresis were prepared by using 1.5% (w/v) Agarose (17 ml) in 0.025 M barbitone buffer , pH 8.2, containing 0.02% NaN_3 and 1.5% polyethylene glycol. The electophoresis conditions involved application of 14 m amps per slide for 1 h as described by Hudson and Hay (1976).

Agarose gels were freed from soluble protein, dried and stained with 0.5% Panceu S in 5% acetic acid. Destaining was performed in 5% acetic acid.

Experimental and Results

Proteolytic digestion of milk fat globules

Washed cream (500 ml) was suspended in 50 mM-Tris-HCl buffer, pH 7.8, (1000 ml) containing 4 mM-$CaCl_2$ at 37°C and 50 mM-Tris-HCl buffer, pH 7.8, (500 ml) containing pronase (150 µg/ml) at 37°C was added. The digestion mixture was incubated for 1 h at 37°C with continuous gentle stirring, cooled on ice and centrifuged (2,300 x g, 1h). The aqueous phase (subnatant) was carefully separated from the fat, concentrated (to 200 ml) by rotary evaporation (at 37°C) and extensively dialysed against distilled water at 4°C. The non-diffusible material was further concentrated (to 60 ml) and centrifuged (100,000 x g, 1h) to remove suspended lipid. The resulting yellow-brown aqueous phase was concentrated (to 25ml) and centrifuged (125,000 x g, 1h) to give a pellet, a lipid layer and a clear solution containing hexose and sialic acid. The clear layer was carefully removed and was divided into 4 ml samples which were stored at -20°C.

Treatment of washed cream in the absence of pronase resulted in the separation of a much less intensely coloured solution which contained almost no soluble sialic acid although small amounts of fat membraneous pellet, presumably resulting from mechanical manipulation of the sample were present in the pronase-free control.

Purification of the pronase-cleaved glycopeptides

The pronase-digested material (4 ml) from the above step was applied to a column (90 cm x 2.5 cm) of Sephadex G-50 (fine) previously equilibrated with water at 22°C. The column was eluted with water at 22°C with a flow rate of 60 ml/h. Fractions (10 ml) were automatically collected and continuously monitored at 280 nm. Individual

fractions were assayed for hexose and sialic acid and a major, included peak was found to contain approximately 60% of the hexose and 80 - 90% of the sialic acid of the initial sample(Fig. 25). Fractions corresponding to the major sialic acid-hexose peak and showing low absorbance at 280 nm were pooled and freeze-dried to give a white fluffy residue (approx. 100 mg) which was dissolved in 0.05 M pyridine acetate buffer, pH 5, (4 ml) and samples (1 ml) were applied to a column (25 cm x 1.5 cm) of DEAE-Sepharose CL-6B previously equilibrated in the same buffer. The column was successively eluted at 22°C with 0.05 M pyridine acetate buffer, pH 5, (100 ml), 0.1 M pyridine acetate buffer, pH 5 (100 ml) and 0.5 M pyridine acetate buffer, pH 5 (100 ml). Fractions (5 ml) were automatically collected and aliquots (0.1 ml) were assayed, after removal of buffer, for hexose, sialic acid and protein (Fig. 26). Elution with 0.05 M pyridine acetate gave two peaks one of which contained sialic acid and both of which contained hexose and protein. Elution with 0.1 M pyridine acetate gave a small peak containing sialic acid with no detectable hexose or protein. Elution with 0.5 M pyridine acetate gave a sharp single peak which contained sialic acid, hexose and protein. Elution with buffer of higher molarity resulted in no more elution of sialic acid and/or hexose-containing protein species. The sialic acid-containing peak eluted with 0.05 M pyridine acetate is referred to as 'sialic acid-poor' (SP) glycopeptide fraction and that eluted with 0.5 M pyridine acetate is referred to as 'sialic acid-rich' (SR) glycopeptide fraction. Fractions corresponding to each fraction were pooled separately and freeze-dried. The residue resulting from freeze-drying the SP glycopeptide fraction was white and fluffy whereas that resulting

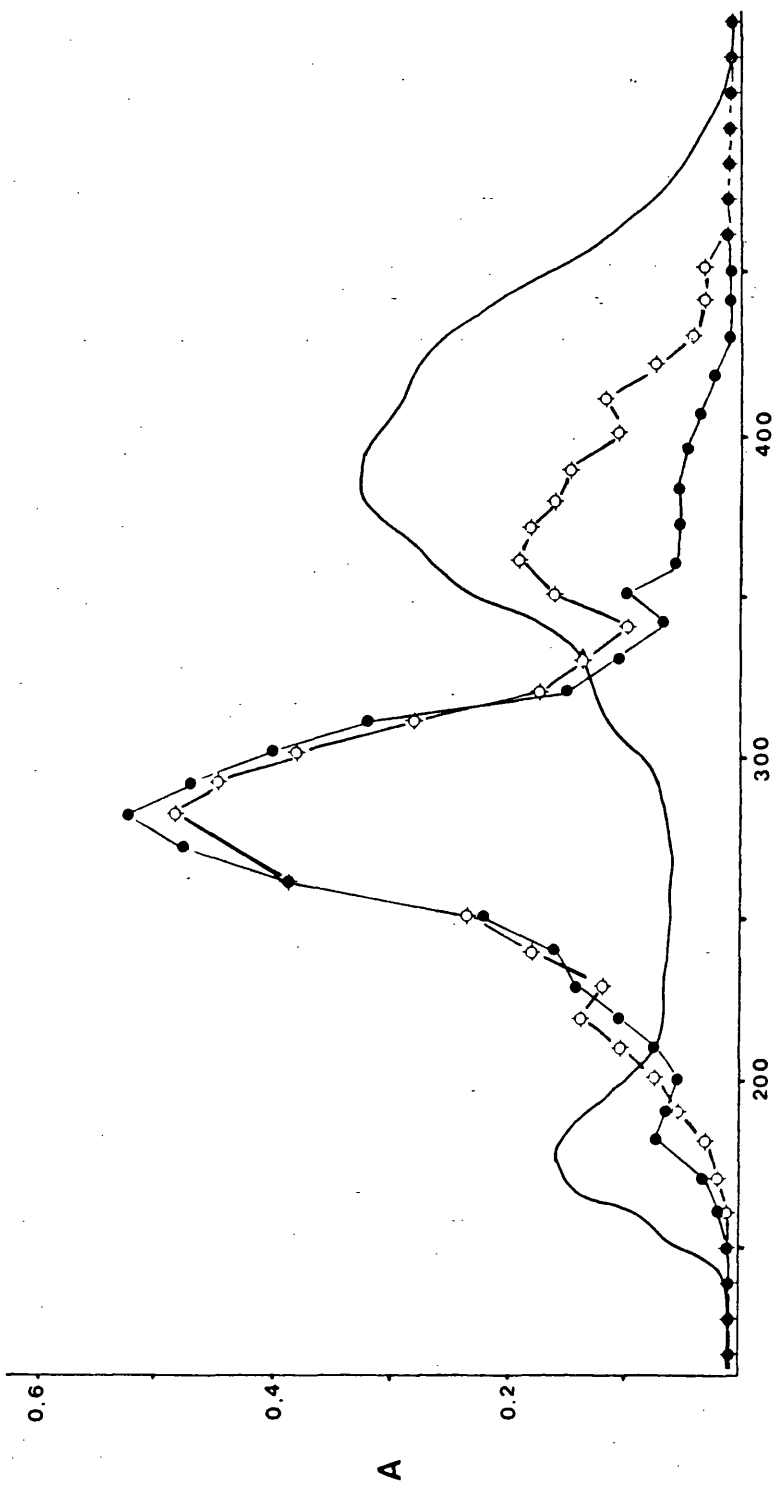


Fig. 25. Fractionation on Sephadex G-50 of pronase cleaved glycopeptides. The column was eluted with water with a flow rate of 60 ml/h. Fractions (10 ml) were collected from which aliquots (0.1 ml) were assayed for hexose (A_{420} ◇) and sialic acid (A_{550} ●). Protein A_{280} ⁻) was continuously monitored using a Cecil 272 spectrophotometer.

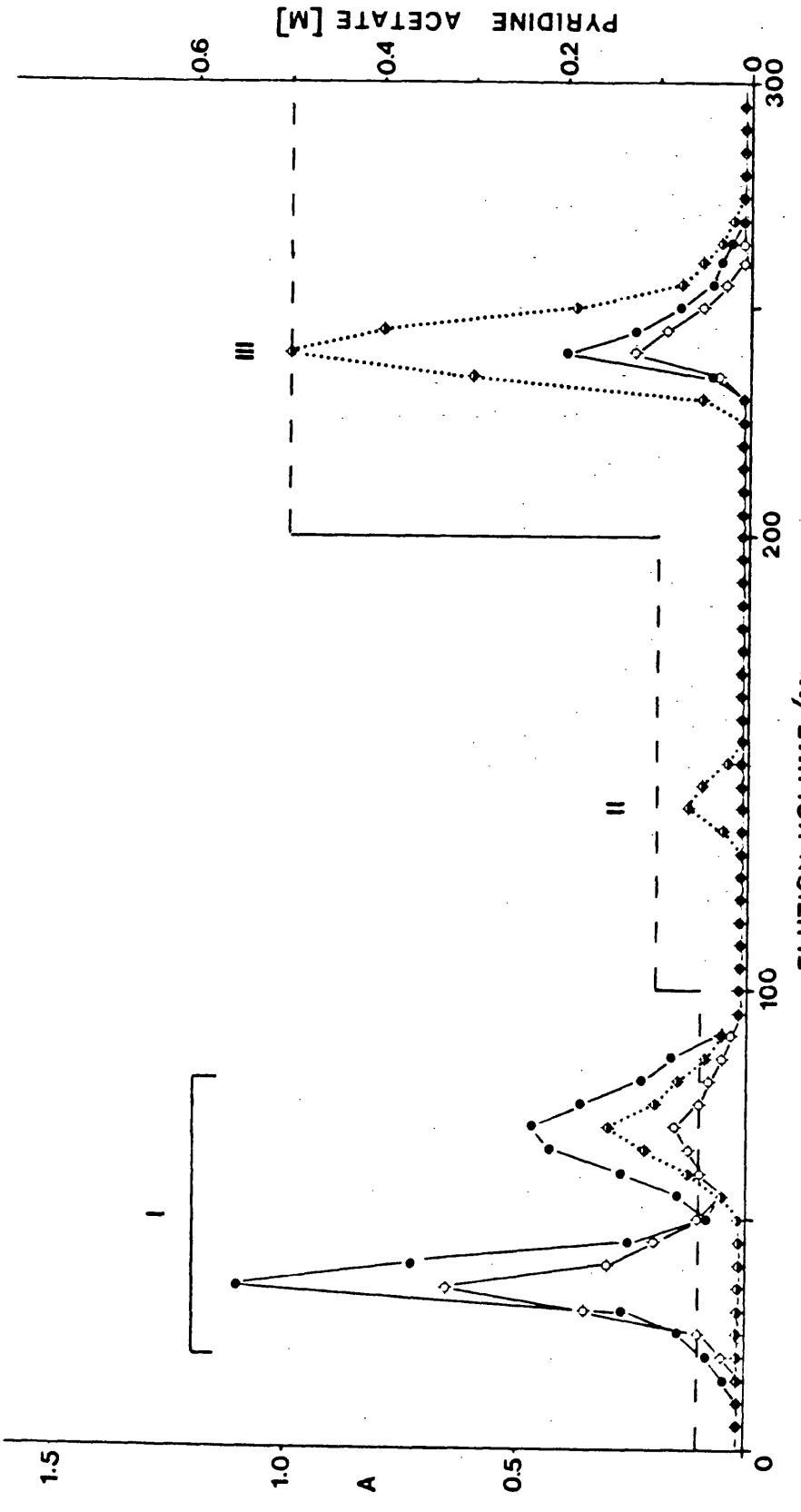


Fig. 26. Fractionation on DEAE Sepharose Cl-6B of the pronase-cleaved glycopeptides. The column was successively eluted with 0.05 M-; 0.1M-; and 0.5M-pyridine acetate, pH 5.0, (100 ml of each) (--). Aliquots (0.1 ml) of fractions (5 ml) were individually assayed for hexose (A_{420} ●), sialic acid (A_{550} ◆) and protein (A_{750} ◇) after removal of buffer.

from SR glycopeptide fraction was a wet brownish residue smelling of pyridine. The freeze-dried materials of both fractions were separately dissolved in 0.1 M acetic acid (1.5 ml) and applied to a column (100 cm x 1.5 cm) of Sephadex G-25 (fine) and eluted at 22°C with 0.1 M acetic acid with a flow rate of 40 ml/h to give in each case a single excluded peak containing sialic acid, hexose and protein (Fig. 27 a, b). Fractions corresponding to the main two peaks were collected as indicated, so as to exclude the tail of each peak (Fig. 27 a,b) and freeze-dried to give a white fluffy residue (approx. 5 mg) which was stored desiccated at -20°C.

Molecular Weight determination of SR and SP glycopeptides

The molecular weight of the SR (Fig. 27a) and SP (Fig. 27b) glycopeptides was estimated by gel filtration on a column (100 cm x 1.5 cm) of Sephadex G-75 by comparison with protein standards. Ovalbumin (mol. wt. 43,000) (8 mg), chymotrypsinogen A (Mol. Wt. 25,000) (5 mg) and ribonuclease A (mol. wt. 13,700) (6 mg) were combined together with blue Dextran (2 mg), dissolved in 0.1 M acetic acid (1.5 ml) and applied to a column of Sephadex G-75 (100 x 1.5 cm). The column was eluted at 22°C with 0.1 M acetic acid with a flow rate of 60 ml/h. Fractions (5 ml) were automatically collected and continuously monitored at 280 nm. The SR (5 mg) and SP (10 mg) glycopeptides were chromatographed separately under the same conditions and fractions (3 ml) were assayed for sialic acid and hexose to give in each case a single symmetrical peak. A plot of mol. wt. versus Kav (Fig. 28) gave a straight line from which the molecular weights of SR and SP glycopeptides were calculated to be 23,500 and 13,000 respectively.

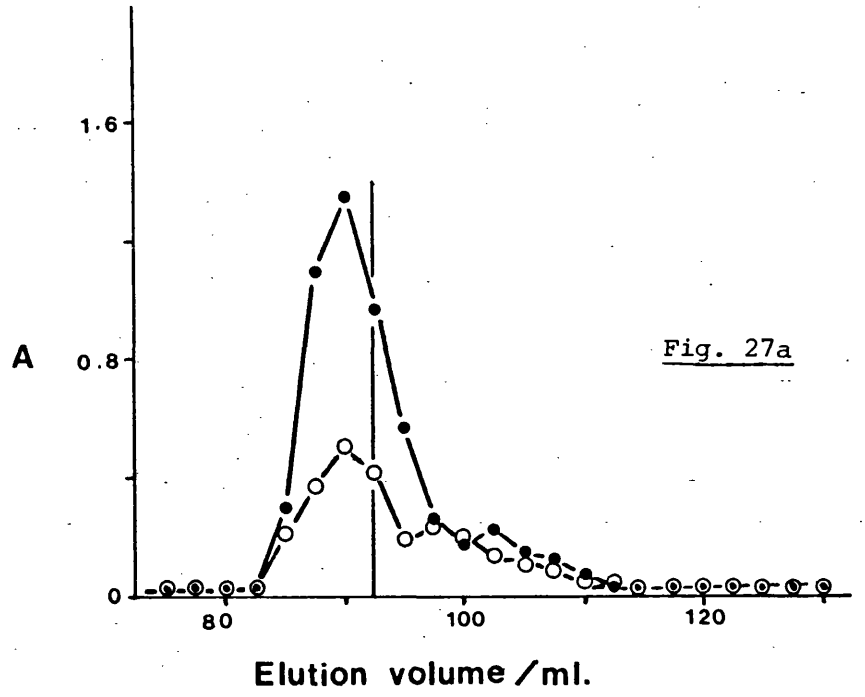


Fig. 27a

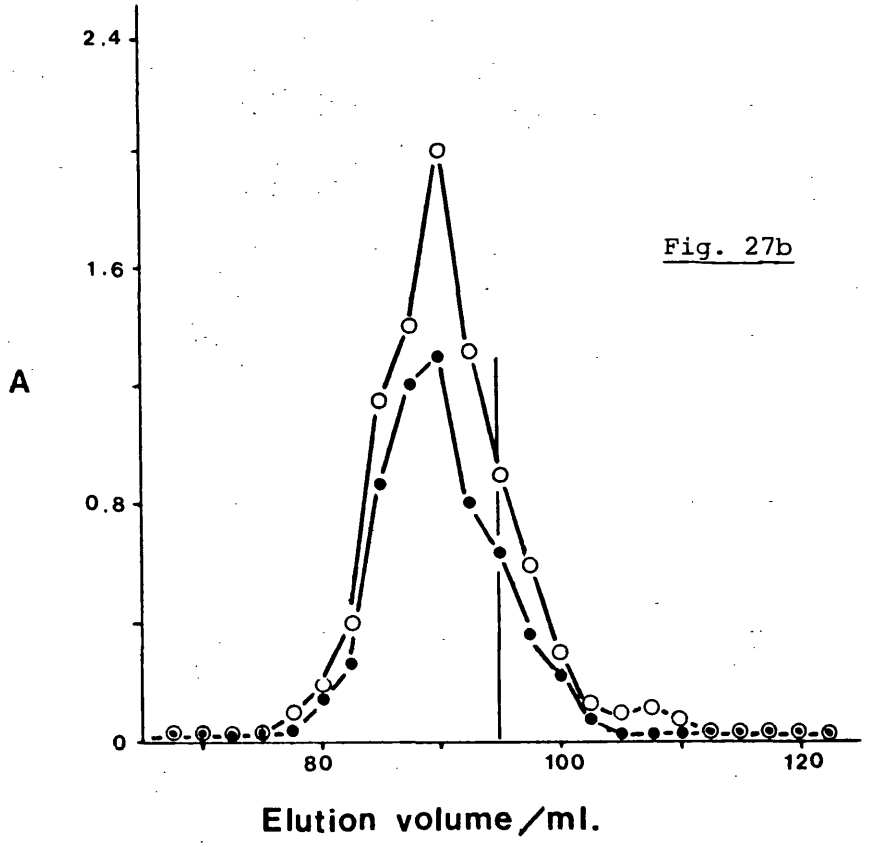


Fig. 27b

Fig. 27. Desalting of the SR glycopeptide (a) and SP glycopeptide (b) on Sephadex G-25. The column was eluted with 0.1M acetic acid. Aliquots (0.1 ml) of fractions (2.5 ml) were individually assayed for hexose (A_{420} o) and sialic acid (A_{550} •).

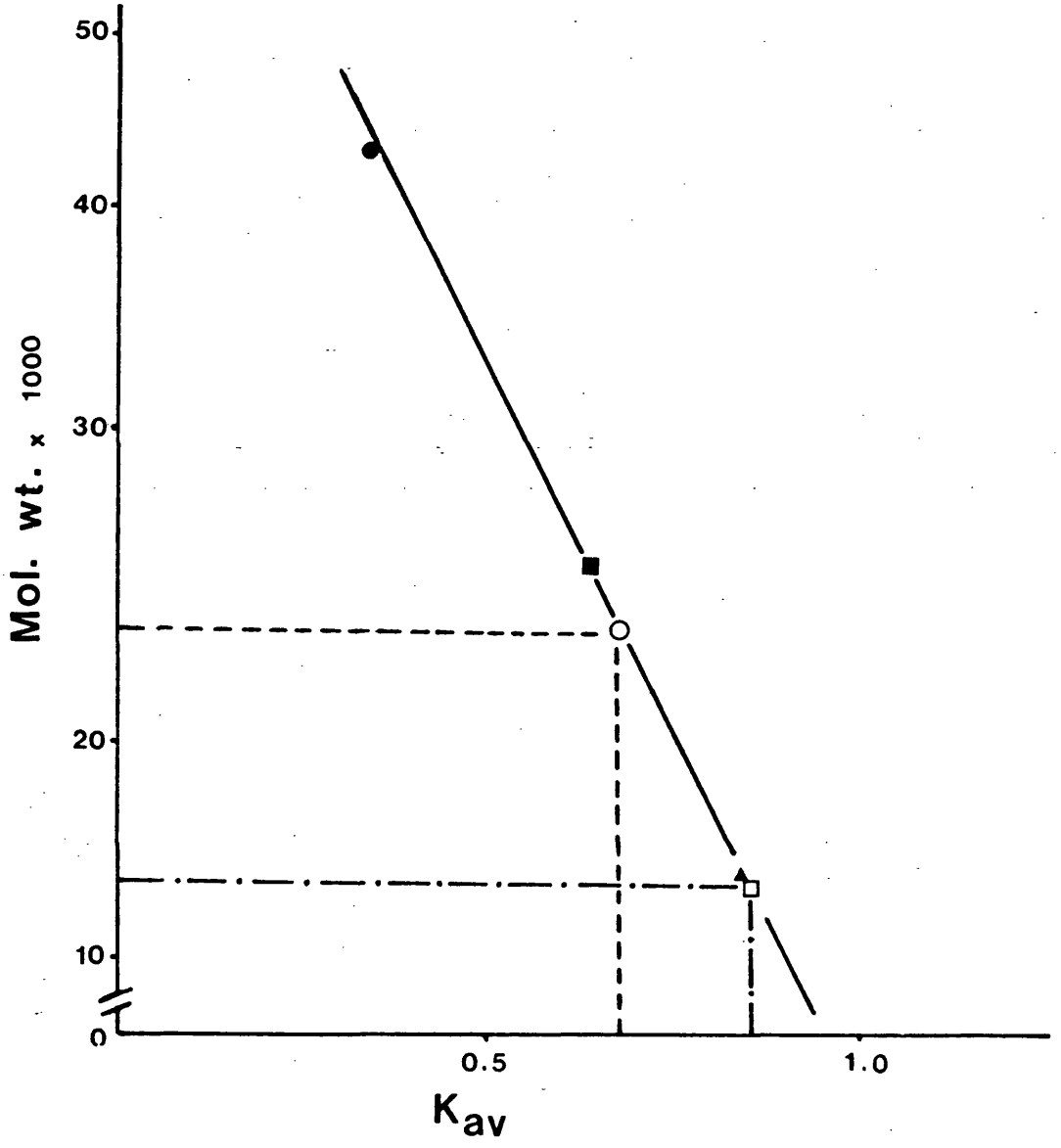


Fig. 28. Calibration plot for Sephadex G-75 column of protein standards (ovalbumin (●), chymotrypsinogen A (■), ribonuclease A (▲)) from which mol. wts. of SR(O) and SP (□) glycopeptides were determined.

$$K_{av} = \frac{v_e - v_o}{v_t - v_o}$$

where v_e = elution volume for the protein
v_o = column void volume
v_t = total bed volume.

The homogeneity of the SR and SP glycopeptides was further tested and their molecular weights were again estimated by disc polyacrylamide gel electrophoresis (PAGE). Protein standards and sialoglycopeptides (SR and SP) samples were prepared for electrophoresis as described by Weber et al., (1972). Electrophoretic mobilities of the protein standards and sialoglycopeptides samples were examined at four gel concentrations (7.5, 10,0, 12.5 and 15.0% acrylamide). Unlike the protein standards, neither the SR nor the Sp glycopeptides were stained with Coomassie Brilliant Blue [0.25% (w/v) in methanol/glacial acetic acid/water (5:7:88, v/v/v)] suggesting that the soluble sialoglycopeptides were not precipitated at the fixation stage and were being washed out of the gel. However, when gels were fixed with TCA/sulphosalycilic acid (30 g; 9 g) in water (186 ml) as described by Vesterberg et al.,(1977) a single sharp band was observed in the case of SR glycopeptide and a single faint band was observed in the case of SP glycopeptide.

Mobility values were calculated according to the following formula:

$$\text{Mobility} = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}} \times 100$$

Table 6 gives the mobility values (average of four experiments) for protein standards, SR glycopeptide and SP glycopeptide at four gel concentrations. Fig. 29 shows plots of log mol. wt. versus mobility for standards. Fig. 30 shows the apparent mol. wt. of the SR and SP glycopeptides (determined from the calibration curves

% acrylamide in gel	IgM (μ-chain) mol.wt. 74,000	IgG (γ-chain) mol. wt. 50,000	IgG (K-chain) mol.wt. 23,000	Ovalbumin mol. wt. 13,700	Native SP-glyco-peptide	Native SR-glyco-peptide
7.5	37.0	50.0	81.0	97.0	72.8	62.8
10	26.0	39.0	66.7	86.2	68.7	60.9
12.5	17.6	28.7	54.3	75.8	66.7	54.2
15	-	20.0	39.0	56.0	50.0	39.5

Table 6. Mobility data for molecular weight standards for native SR and native SP glycopeptides obtained by SDS PAGE at four acrylamide gel concentrations. Mobilities were calculated according to the following formula:

$$\text{Mobility} = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}} \times \frac{\text{length of gel before staining}}{\text{length of gel after staining}} \times 100$$

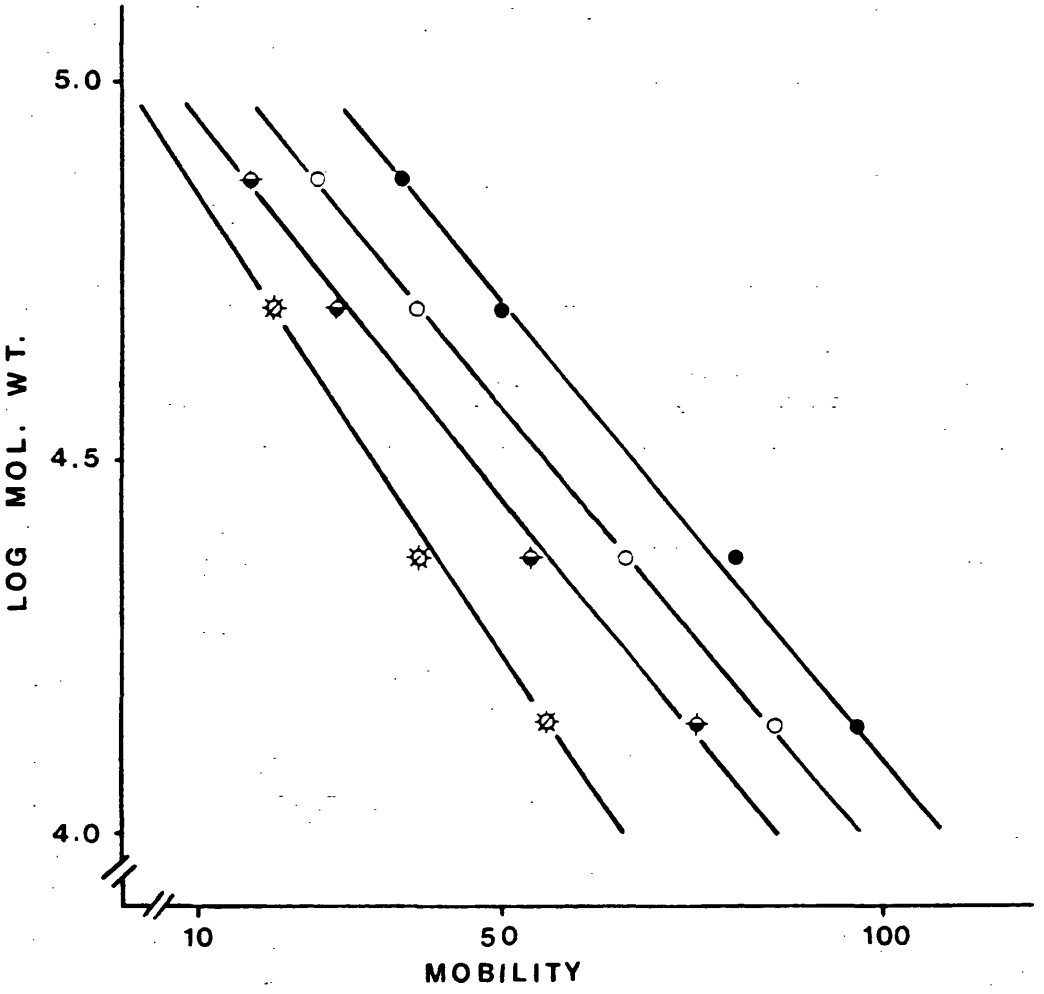


Fig. 29. The calibration plots for four acrylamide gel concentrations (7.5%●, 10.0% ○, 12.5%◈, 15%✡). Substitution of mobility data for SR and SP glycopeptides (Table 6) in this graph allows the estimation of their log. molecular weight on any given gel concentration.

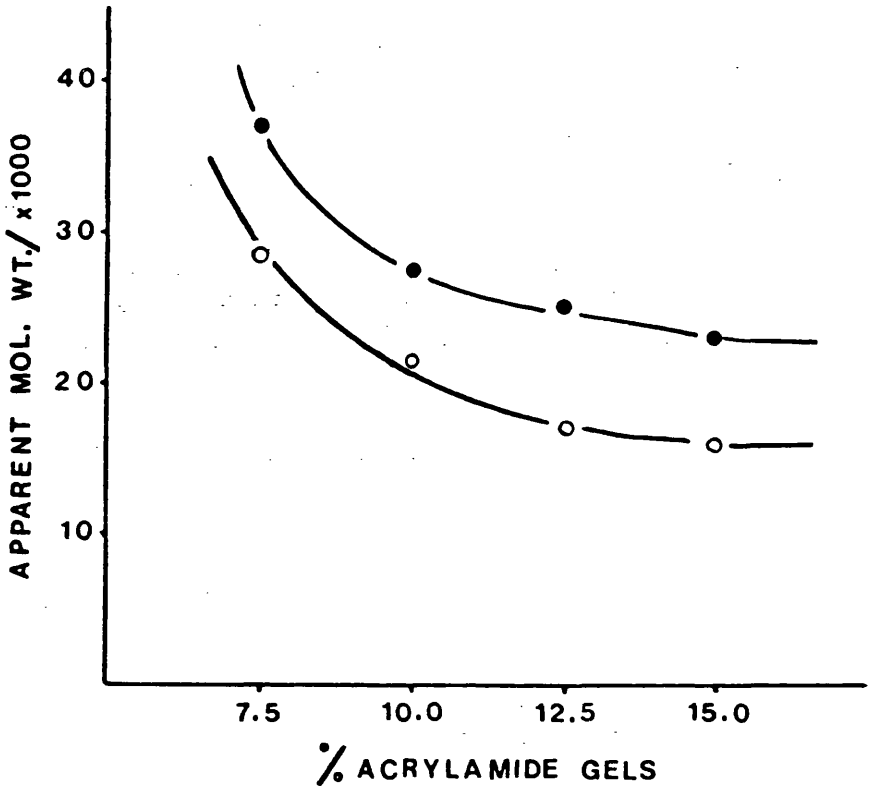


Fig. 30. Estimation of asymptotic minimal apparent molecular weights of SR (• — •) and SP glycopeptide (o — o) at four gel concentrations.

of (Fig. 29), plotted against acrylamide gel concentrations according to the procedure of Segrest and Jackson (1972). The asymptotic minimal molecular weights of SR and SP glycopeptides, determined according to this procedure are approximately 23,000 and 17,000 respectively.

Desialylation of sialoglycopeptides

The removal of sialic acid from purified SP and SR glycopeptide fractions was carried out by mild acid hydrolysis. Sialoglycopeptide samples (5 mg) were incubated at 80°C for 1 h in 0.2 N H_2SO_4 (1.5 ml) The hydrolysate was passed through a column (100 cm x 1.4 cm) of Sephadex G-25 (fine) to separate free sialic acid from the residual desialylated glycopeptides. The column was eluted at 22°C with a 0.1 M acetic acid with a flow rate of 40 ml/h. Fractions (3 ml) were automatically collected and assayed for sialic acid and hexose, giving, in both cases, a major excluded hexose-containing peak together with an included peak of free sialic acid (Fig. 31).

Carbohydrate composition of the sialic acid-rich glycopeptide

The purified sialoglycopeptide (200µg) in 2N-TFA (50 µl) containing perseitol (20 µg), as an internal standard, was hydrolysed at 121°C for 1 h and the hydrolysate was freed from acid in a stream of N_2. The free monosaccharides were then converted to alditol acetate derivatives as described in the Materials and Methods section. The alditol acetate derivatives were dried and the residue was dissolved in dichloromethane (100 µl). Samples (0.5 - 1.0 µl) of the dichloromethane solution were injected directly into the gas chromatograph. The chromatographic trace obtained from injections onto the column of OV-225 is shown in Fig. 32 and that obtained from injections into the column of OV-17 shown in Fig. 33. Peaks

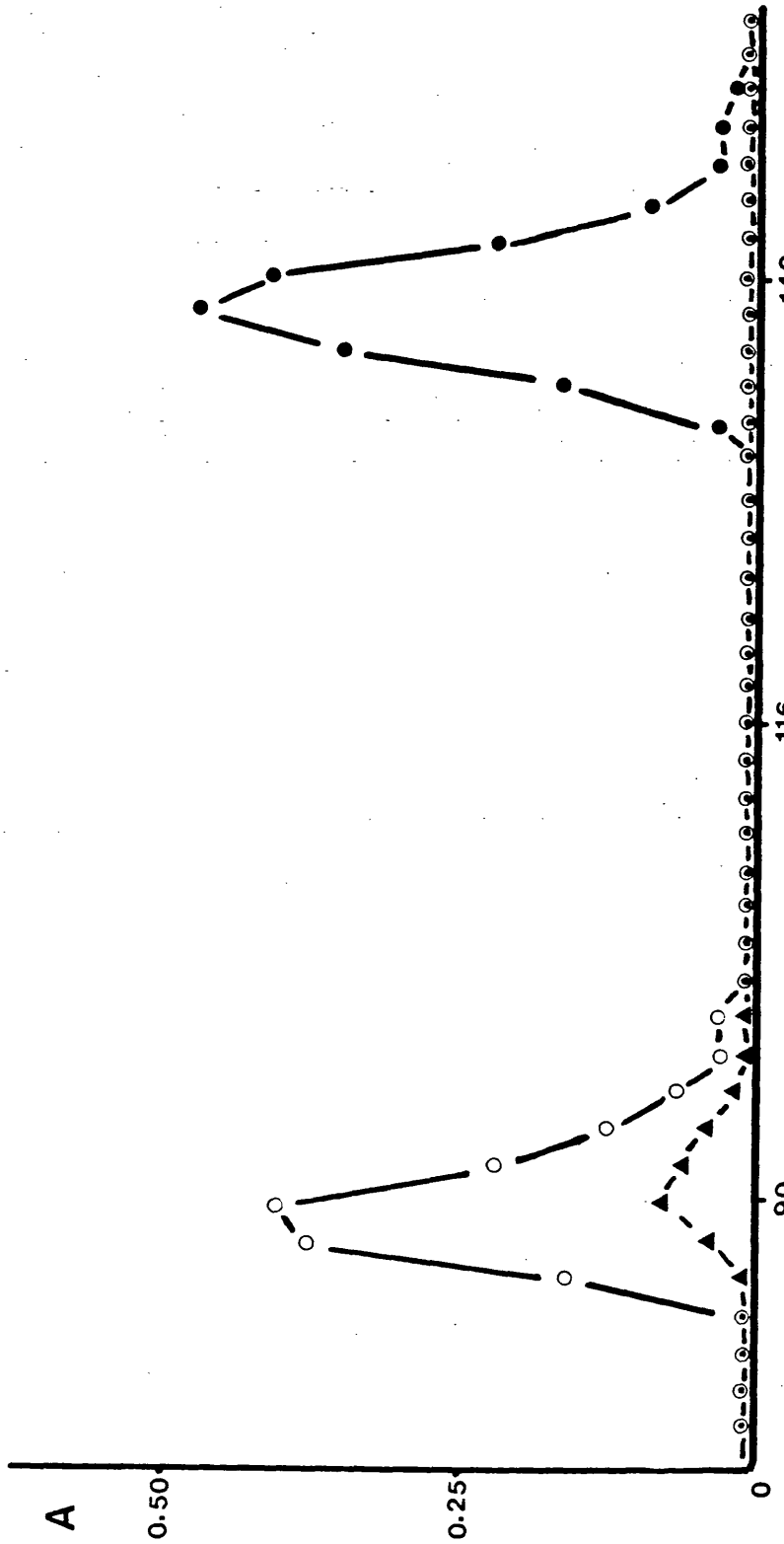


Fig. 31. Separation of desialylated SR glycopeptide from free sialic acid after mild acid hydrolysis. The Sephadex G-25 column was eluted with 0.1M-acetic acid. Aliquots (0.1 ml) of fractions (2 ml) were individually assayed for hexose (A_{420} ○), sialic acid (A_{550} ●) and protein (A_{750} ▲).

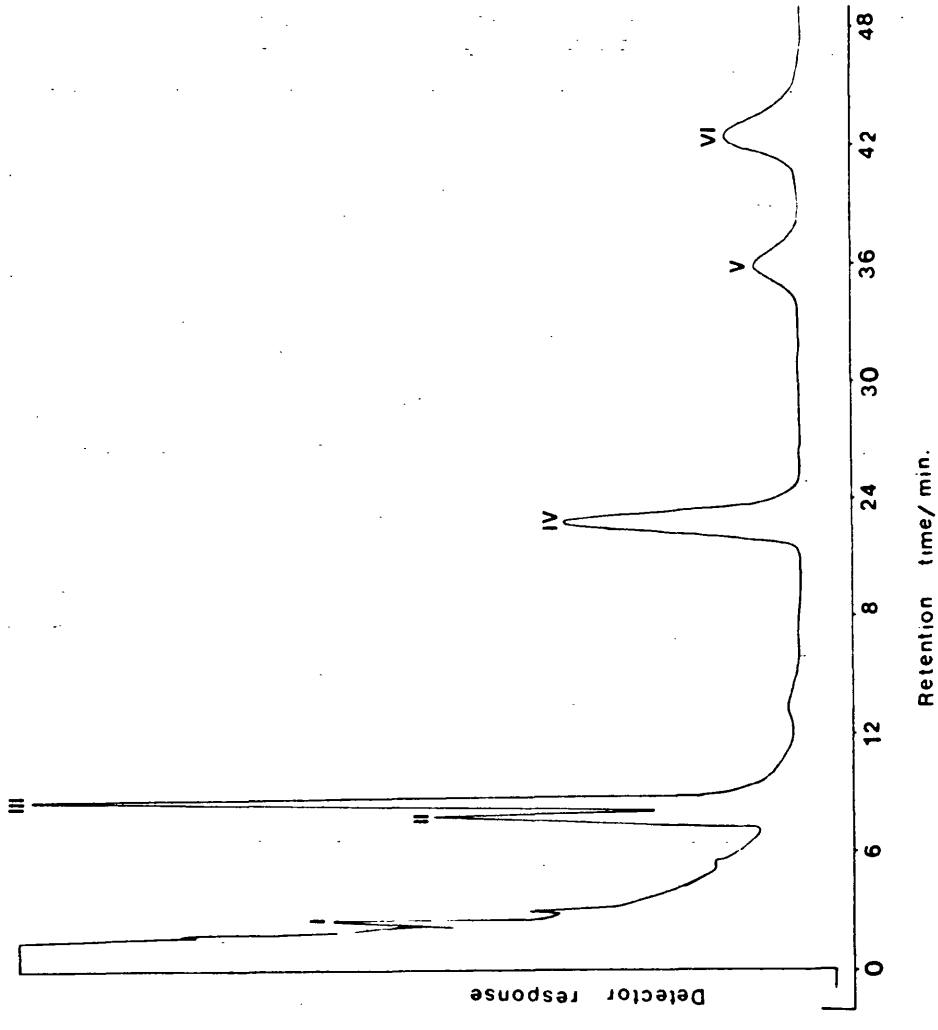


Fig. 32. Gas chromatogram of alditol acetate derivatised monosaccharides released during acid hydrolysis of native SR glycopeptide. Peaks I - VI correspond to: L-fucose; D-mannose; D-galactose; Perseitol (internal standard); N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Chromatography was performed isothermally at 200^{o}C on a column of 3% OV-225.

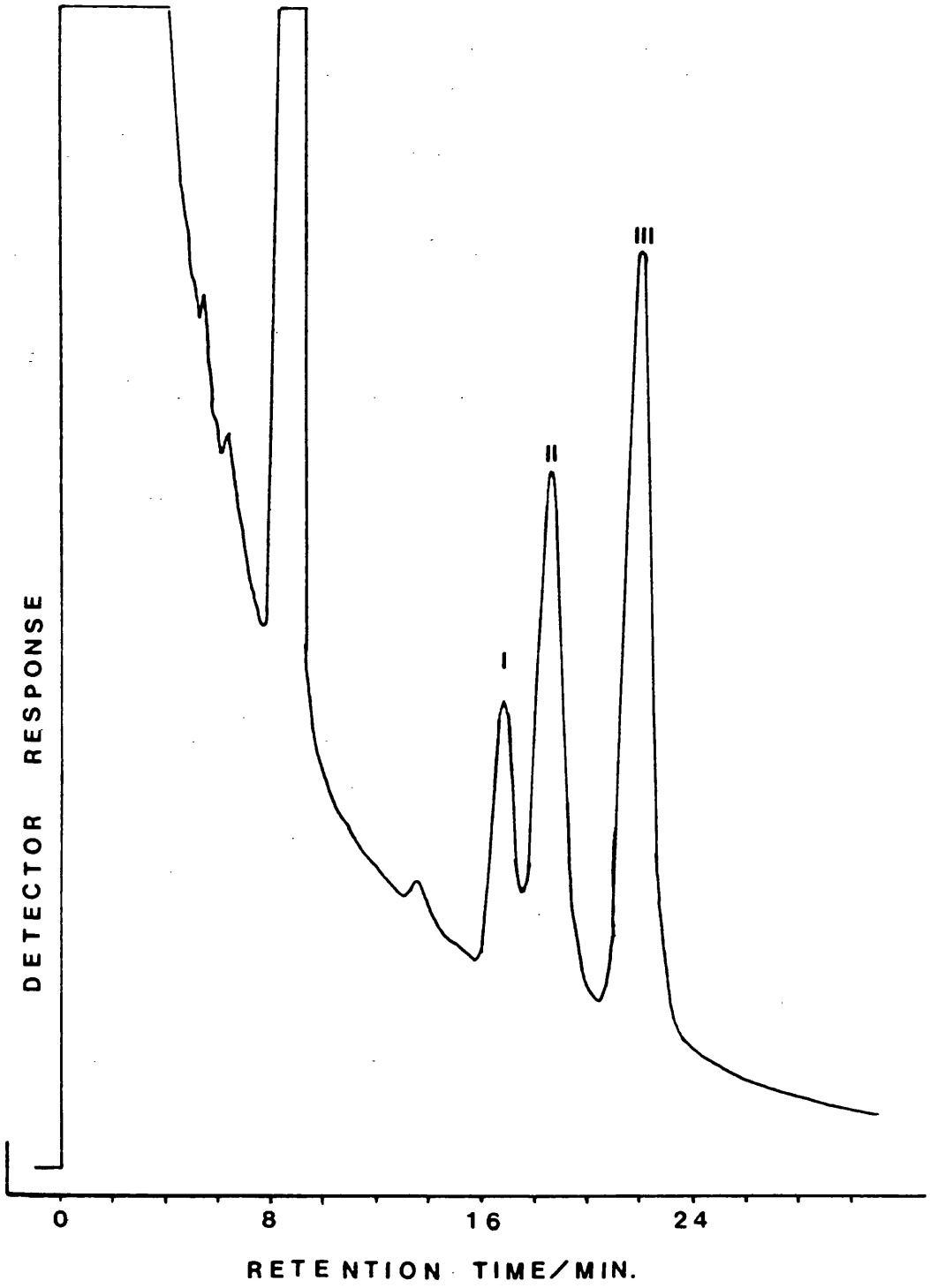


Fig. 33. Gas chromatogram of alditol acetates from hydrolysis of SR glycopeptide. Peaks I, II and III represent N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and perseital respectively. Chromatography was performed isothermally at 205°C on a column of 3% OV-17.

I, II, III, IV, V and VI in Fig. 32 were identified, by comparison with authentic standards, as the alditol acetates of L-fucose, D-mannose, D-galactose, perseitol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Peaks I, II and III in Fig. 33 were similarly identified as the alditol acetates of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and perseitol respectively. The relative average concentrations of the constituent sugars are shown in Table 7. The sialic acid content was estimated colorimetrically (P. 67). Derivatives of the desialylated glycopeptide (P. 83) obtained by the same procedure gave the same pattern of sugars (apart from sialic acid) in the same relative proportions. This control experiment showed that charring, which was evident following hydrolysis of the sialoglycopeptide, but not of the desialylated product, did not influence the apparent monosaccharide composition.

Alkaline borohydride treatment of the sialic acid-rich glycopeptide

The purified sialoglycopeptide (25 mg), excluded from Sephadex G-25 (Fig. 27a) was dissolved in 0.05 M-NaOH containing 1.0 M-sodium borohydride (25 ml) and the solution was incubated for 14h in the dark at 50°C in a sealed container under N_2. The mixture was then cooled in ice and excess borohydride was neutralised by adjusting the solution to pH 6.0 by the careful addition of 25% (v/v) acetic acid. The solution was then passed through Sephadex G-25 (fine) column (2.5 cm x 90 cm) to remove the salts. The salt-free mixture of the residual peptide and the alkali cleaved fragments was freeze-dried and the resultant residue was dissolved in 0.05 M pyridine acetate buffer, pH 5.0 (1.5 ml) and applied to a DEAE-Sephadex A-25 column (25 x 1.5 cm) previously equilibrated with the

Source	Monosaccharide components expressed as μmol/100 mg peptide					
	L-fucose	D-mannose	D-galactose	N-acetyl-D-glucosamine	N-acetyl-D-galactosamine	N-acetyl neuraminic acid
Native sialoglycopeptide	7.19	25.94	58.55	15.28	32.57	43.55
Alkaline borohydride treated sialoglycopeptide	5.0	18.88	26.11	14.93	15.38	10.88
Periodate treated sialoglycopeptide	trace	16.66	41.66	18.55	29.53	0.00
T. foetus treated sialoglycopeptide	8.23	28.88	11.66	18.55	14.47	0.00

Table 7. Composition of the amounts of individual monosaccharides present in the native, alkaline borohydride, periodate and T. foetus treated sialic acid rich (SR) glycopeptides. Values expressed for hexoses and hexosamines were calculated from gas chromatographic data as described in the Materials and Methods Section. N-acetylneuraminic acid was estimated colorimetrically by the modified thiobarbituric acid of Aminoff (1961).

same buffer. The column was eluted with 0.05 M pyridine acetate buffer, pH 5.0 (100 ml) followed by a linear gradient (0.5 - 0.65 M) of pyridine acetate buffer, pH 5.0(200 ml) with a flow rate of 40 ml/h. Fractions (5 ml) were collected and aliquots (0.1 ml) were assayed for hexose, sialic acid and protein, after removal of the volatile buffer as described before.

The elution profile obtained (Fig. 34) showed three distinct peaks (I, II and III). Peak I, which was eluted with the starting buffer, was found to contain hexose,sialic acid and protein. Peak II was a broad peak of two major components both of which contained hexose, sialic acid and relatively low levels of protein. Peak III, on the other hand, was a single symmetrical peak and contained hexose and sialic acid with no detectable protein. Fractions corresponding to each peak were pooled and freeze-dried. The freeze-dried material corresponding to Peak I was dissolved in 0.1 M acetic acid (1 ml) and chromatographed on a column (100 x 1.5 cm) of Sephadex G-25 (fine) to give a single excluded peak containing high levels of hexose and relatively low levels of sialic acid and protein (Fig. 35). Fractions corresponding to this peak were pooled and freeze-dried to give a fluffy residue (approx. 2.5 mg). Samples (200 µg) of the freeze-dried material were hydrolysed with TFA and converted to alditol acetate derivatives as described for the native sialoglycopeptide. The gas chromatographic trace obtained from injections onto the column of OV-225 is shown in Fig. 36. Peaks I, II, III, IV, V and VI in Fig. 36 were identified by comparison with authentic standards as the alditol acetates of L-fucose, D-mannose, D-galactose, perseitol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Relative concentrations of the constituent sugars are

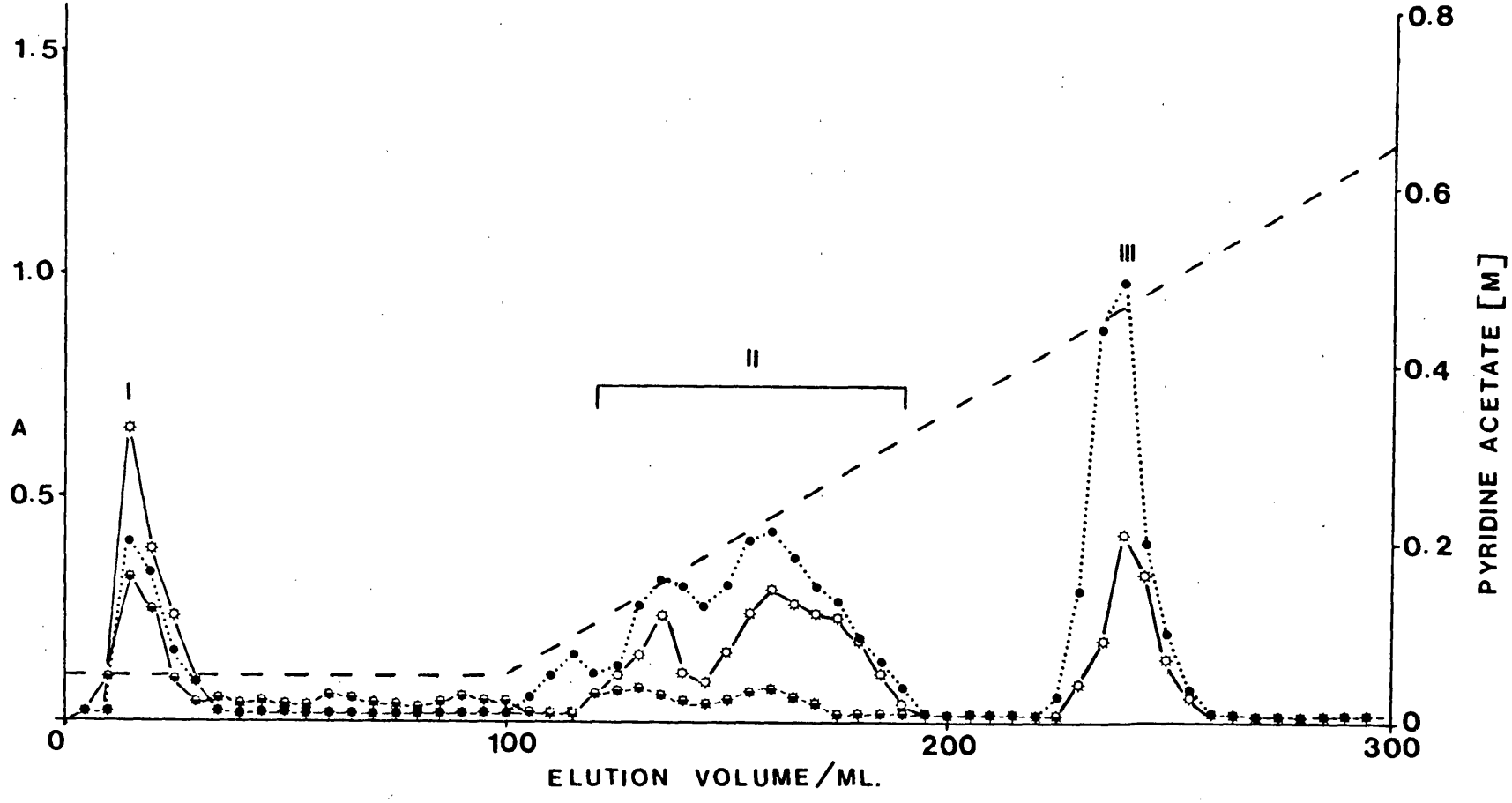


Fig.34. Fractionation on DEAE Sephadex A_{25} of the products resulting from alkaline borohydride treatment of the SR glycopeptide. The column was eluted with 0.05M pyridine acetate buffer, pH 5.0, (100 ml), followed by a linear gradient of 0.05-0.65M pyridine acetate buffer (200 ml --) pH 5.0. Aliquots (0.1 ml) of fractions (5 ml) were individually assayed for hexose (A_{420} ☼), sialic acid (A_{550} ●) and protein (A_{750} ✵) after removal of the buffer.

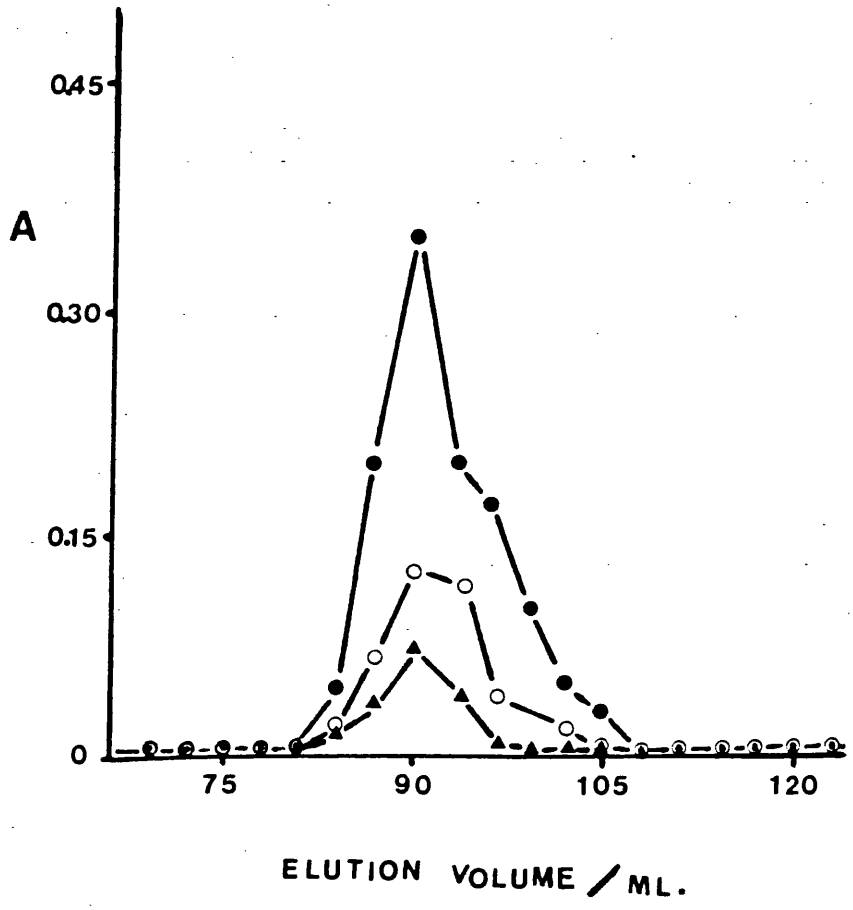


Fig. 35. Desalting on Sephadex G-25 of fractions corresponding to peak I (Fig. 34). The column was eluted with 0.1 M acetic acid. Aliquots (0.1 ml) of fractions (3 ml) were individually assayed for hexose (A_{420} ●), sialic acid (A_{550} O) and protein (A_{750} ▲).

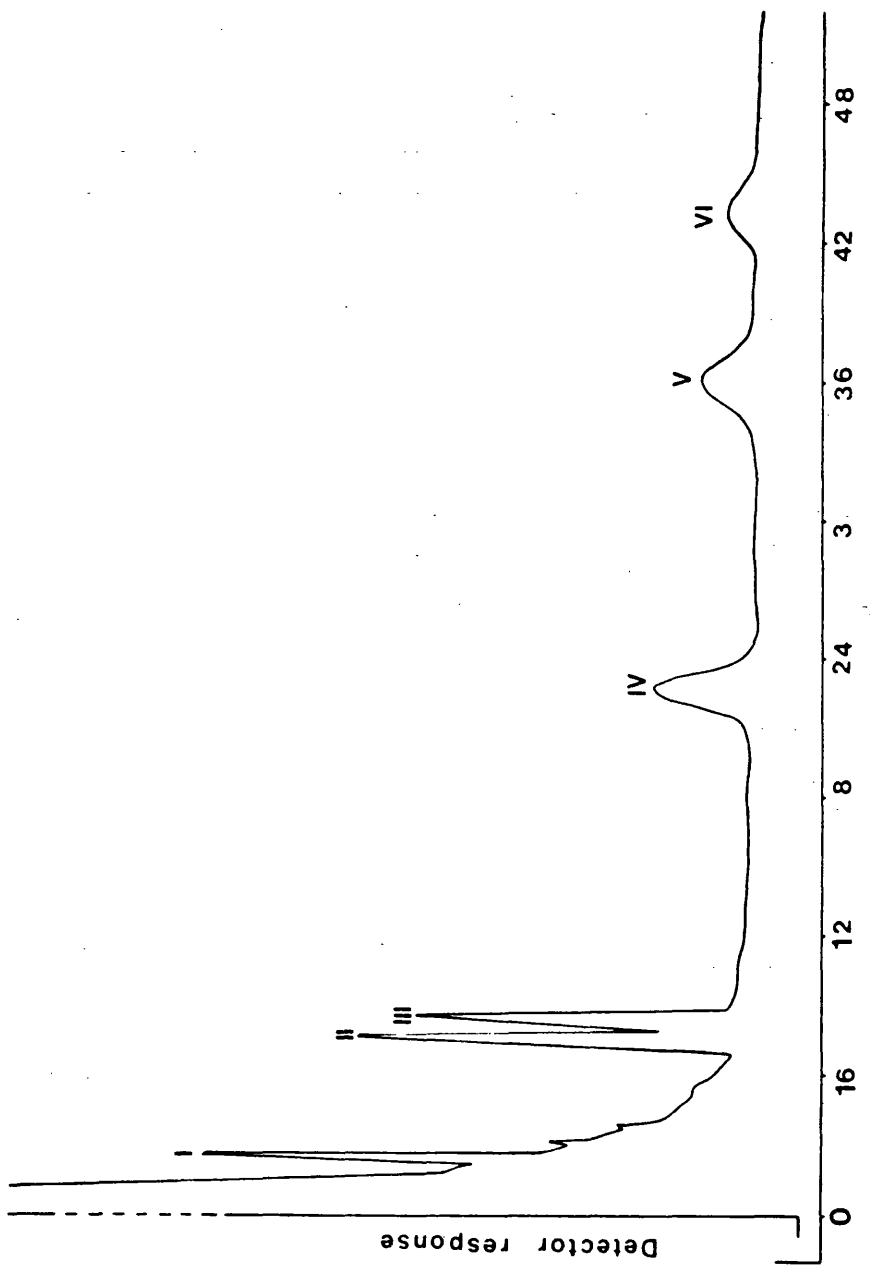


Fig. 36. Gas chromatograph of alditol acetates from hydrolysis of alkaline borohydride treated SR glycopeptide (Fig. 35). For chromatography conditions and identity of peaks I - VI see Fig. 32.

shown in Table 7.

Samples (200 µg) corresponding to peak II and III in Fig. 34 were hydrolysed with TFA and converted to alditol acetates. While the chromatographic trace of peak II was found to be similar to that of the residual glycopeptide (peak I, Fig. 34), indicating relatively high contaminaions of the latter peak, the trace of peak III was found to contain only D-galactose and N-acetyl-D-galactosamine (Fig. 37). together with sialic acid. The molar ratio of D-galactose: N-acetylgalactosamine :sialic acid, in this peak was found to be 1:1:2 respectively.

Periodate oxidation of the sialic acid-rich glycopeptide

Samples of the purified desialylated glycopeptide (10 mg) in 0.05 M sodium acetate buffer, pH 4.5 (2.5 ml) containing 50 mM sodium metaperiodate were incubated for 14 h at 4°C under an atmosphere of N_2 in the dark. Excess periodate was destroyed by addition of 20 mM ethylene glycol (1.5 ml) and the mixture was passed through a column (25 x 1.5 cm) of Sephadex G-25 to remove the salt. The column was eluted at 22°C with 0.1 M acetic acid with a flow rate of 40 ml/h to give an excluded peak containing hexose and protein and an included salt-containing peak. Fractions corresponding to the excluded peak were pooled and freeze-dried. Samples (200 µg) of the freeze-dried material were hydrolysed with TFA and converted to alditol acetates as described for the native glycopeptide. The chromatographic trace obtained from injections onto the column of OV-225 is shown in Fig. 38 and that obtained from injections onto the OV-17 column is shown in Fig. 39.

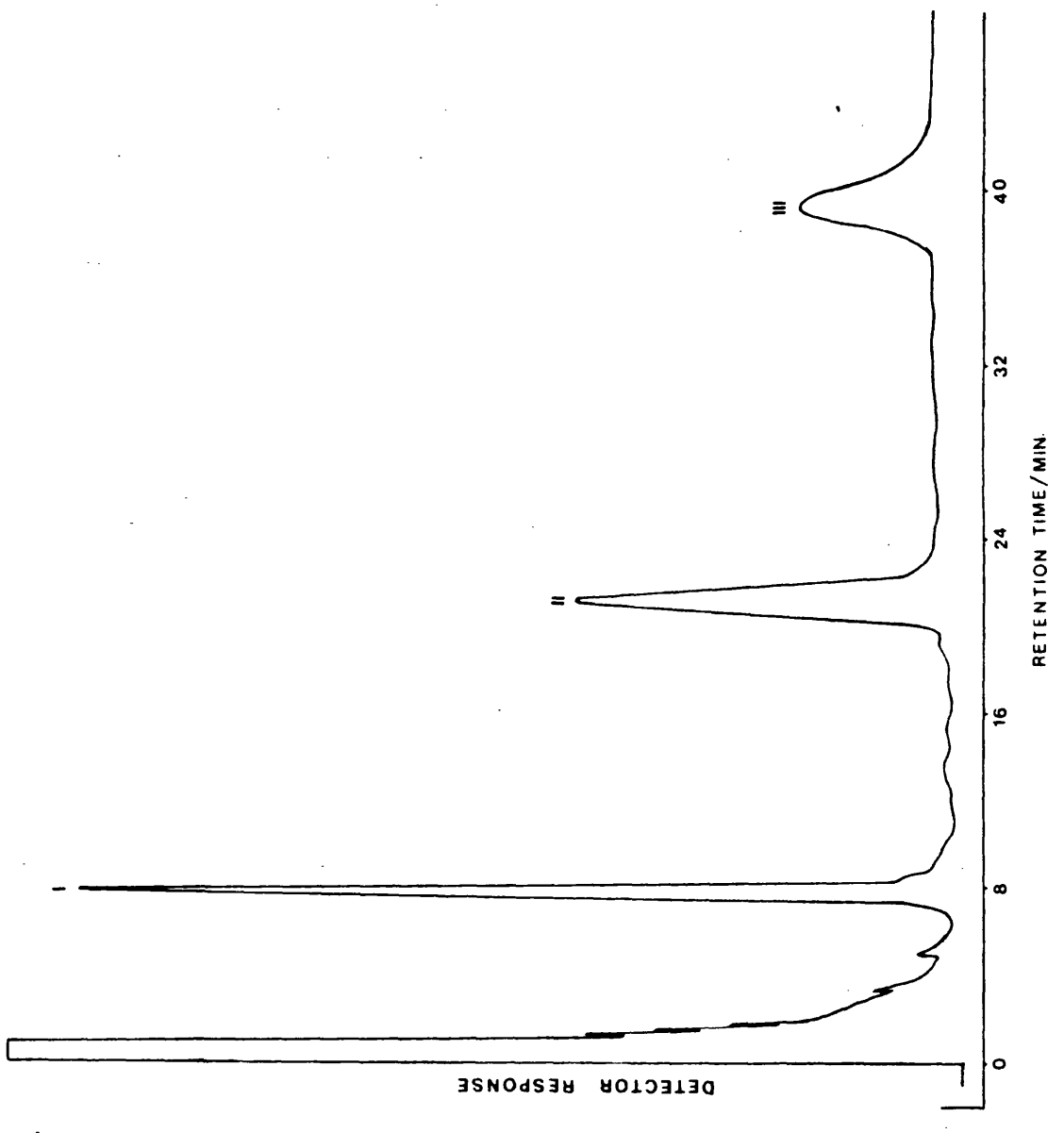


Fig. 37. Gas chromatogram of alditol acetates from hydrolysis of peak III (Fig. 34). Peaks I - II represent D-galactose perseitol and N-acetyl-D-galactosamine. Column and conditions as described in Fig. 32.

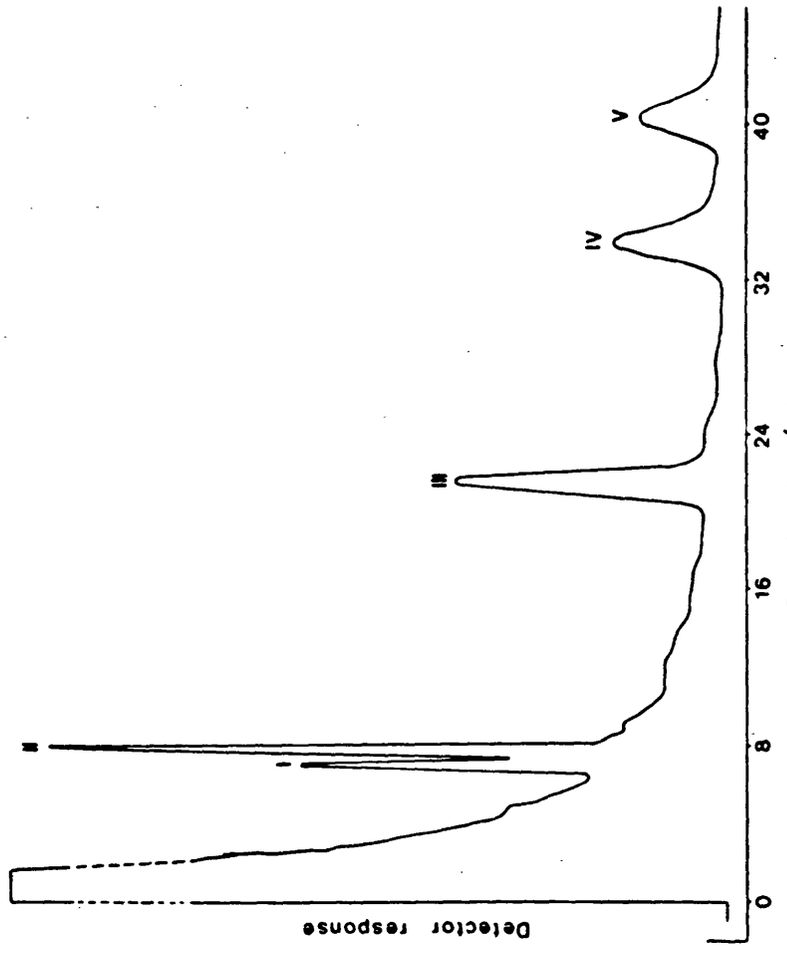


Fig. 38. Gas chromatogram of alditol acetates from hydrolysis of the periodate treated SR glycopeptide. Peaks I - V correspond to: D-mannose, D-galactose, perseitol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Chromatography was performed isothermally at 200°C on a column of 3% OV-225.

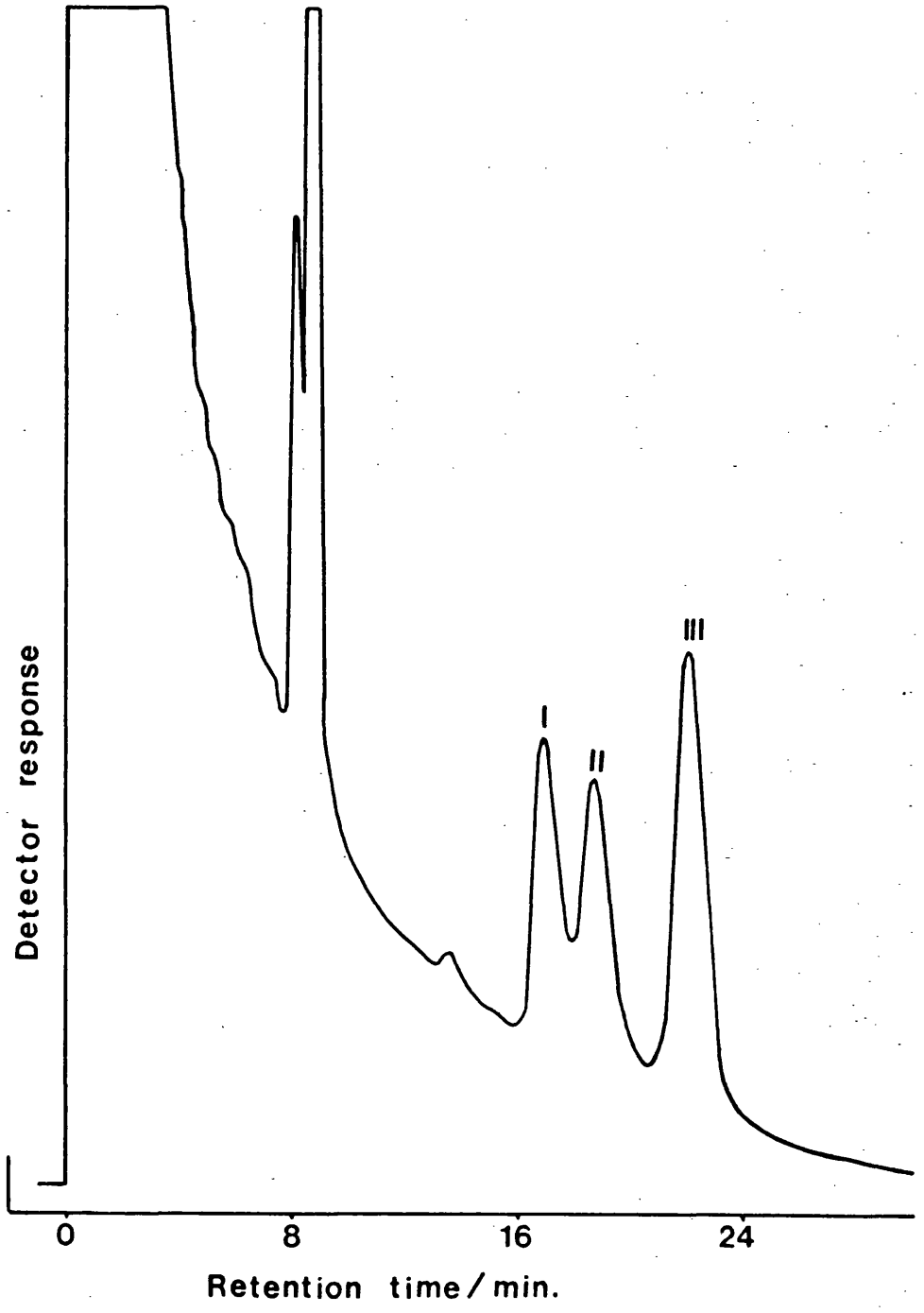


Fig. 39. Gas chromatogram of alditol acetates from hydrolysis of periodate treated SR glycopeptide. For chromatography conditions and identity of peaks I -III see Fig. 33.

Peaks I, II, III, IV and V in Fig. 38 were identified, as described before, as the alditol acetates of D-mannose, D-galactose, perseitol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Peaks I, II and III in Fig. 39 were similarly identifeid as the alditol acetates of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and perseitol respectively. Relative concentrations of constituent sugars of the periodate-treated SR glycopeptide are shown in Table 7. Control experiments performed by omitting the periodate step showed that the carbohydrate content of the glycopeptide was essentially the same as that for the native glycopeptide (Table 7). As can be seen from Table 7, approximately 30% of the galactose and mannose were destroyed by periodate. N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were largely protected from the action of periodate although about 8% of the latter was destroyed.

Treatment of the sialic acid-rich glycopeptide with mixed glycosidases from Trichemonase foetus

The glycosidase extract from T. foetus has been found (Watkins, 1966a; Westwood et al, 1976) to contain fucosdiase, galactosidase and hexosaminidase. The purified desialylated SR glycopeptide (10 mg) was incubated with T. foetus extract (5 mg) in 100 mM potassium phosphate buffer, pH 6.4, (2.5 ml) at 37^{o}C for 4h. The mixture was then passed through a Sephadex G-25 (fine) column (100 x 1.5 cm). The column was eluted with 0.1 M acetic acid with a flow rate of 40 ml/h. Fractions (3 ml) were automatically collected and continuously monitored at 240 nm and aliquots (0. 1ml) were assayed for hexose to give an excluded hexose peak and an included peak of free sugars. (Fig. 40). Fractions corresponding to the excluded peak were pooled and freeze-dried to give a white fluffy residue (approx. 5 mg).

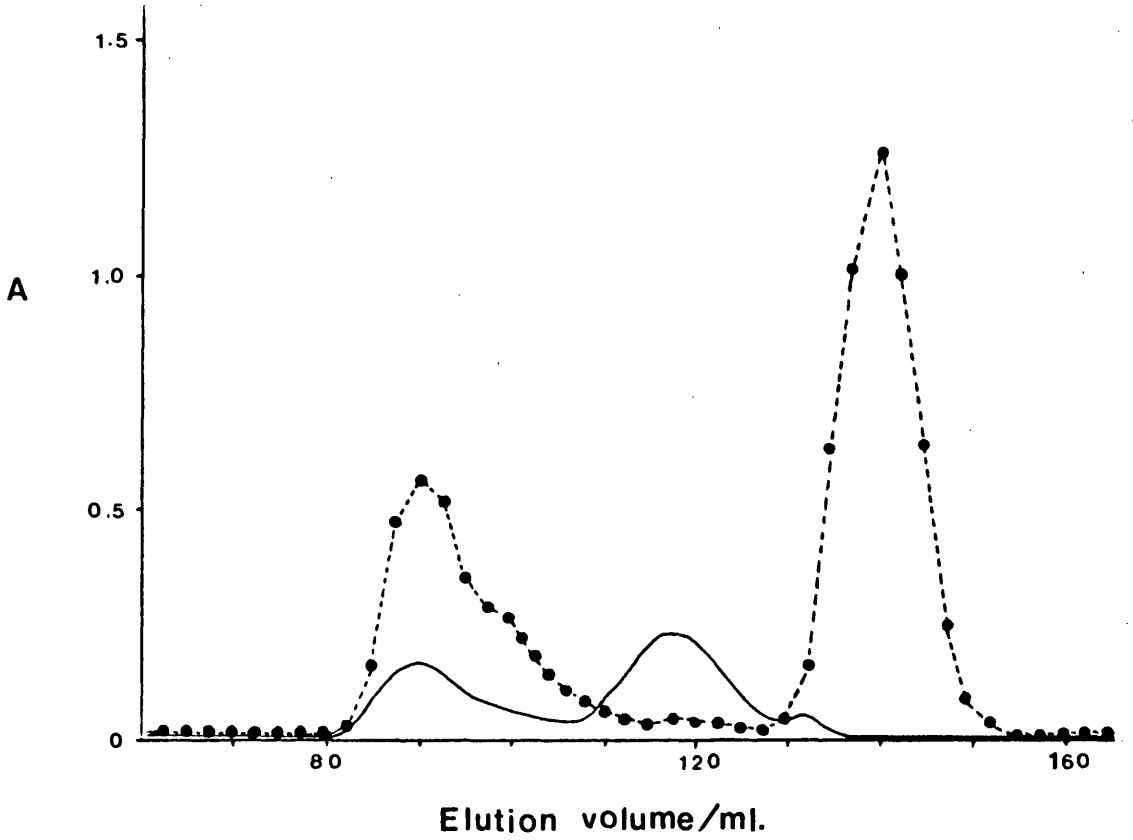


Fig. 40. Desalting on Sephadex G-25 of the T. foetus treated SR glycopeptide. The column was eluted with 0.1 M acetic acid. Aliquots (0.1 ml) of fractions (2.5 ml) were individually assayed for hexose (A_{420} ●), Protein (A_{240} -) was monitored using a Cecil 272 spectrophotometer.

Samples (200 µg) were hydrolysed with TFA and converted to alditol acetates as described for the native glycopeptides. The chromatographic trace obtained from injection of the T. foetus treated SR glycopeptide onto the column of OV-225 is shown in Fig. 41. Peaks I, II, III, IV, V, VI and VII in Fig. 41 were identified by comparison with authentic standards as the alditol acetates of L-fucose, D-mannose, D-galactose, D-glucose, perseitol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Relative concentrations of the constituent sugars are shown in Table 7. Dialysis (against 0.1 M acetic acid or water) of the T. foetus treated SR glycopeptide to remove the released monosaccharide residues was found to be as effective as chromatography on Sephadex G-25. The non-diffusible material showed essentially the same chromatographic trace and carbohydrate composition as that of the excluded peak in Fig. 40. Control experiments carried out by replacing the enzyme with buffer alone or with heat inactivated (100°C, 10 min) enzyme showed that the carbohydrate content was similar to that of the native glycopeptide.

Carbohydrate composition of the sialic acid-poor glycopeptide

The purified sialoglycopeptide (200 µg) was hydrolysed with TFA and converted to alditol acetate derivatives as described for the SR glycopeptide. The chromatographic trace obtained from injections of the derivatised material onto the OV-225 column is shown in Fig. 42 and that obtained from injections onto the column of OV-17 is shown in Fig. 43. Peaks I, II, III, IV, V and VI in Fig. 42 were identified by comparison with authentic standards as the alditol acetates of L-fucose, D-mannose, D-galactose, perseitol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Peaks I

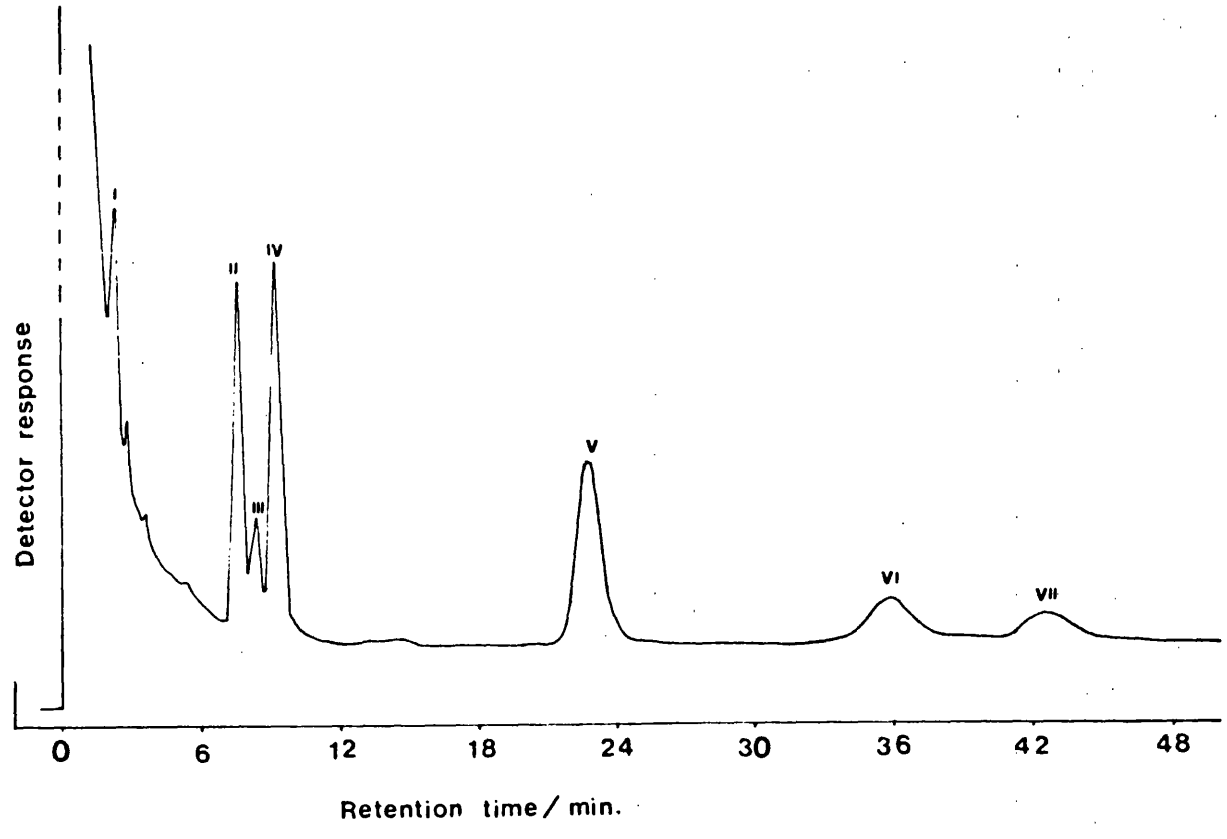


Fig. 41. Gas chromatogram of the alditol acetates from hydrolysis of T. foetus treated SR glycopeptide, Peaks I - VII correspond to: L-fucose, D-mannose, D-galactose, D-glucose, perseitol, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine respectively. Column and chromatography conditions are as described in Fig. 32.

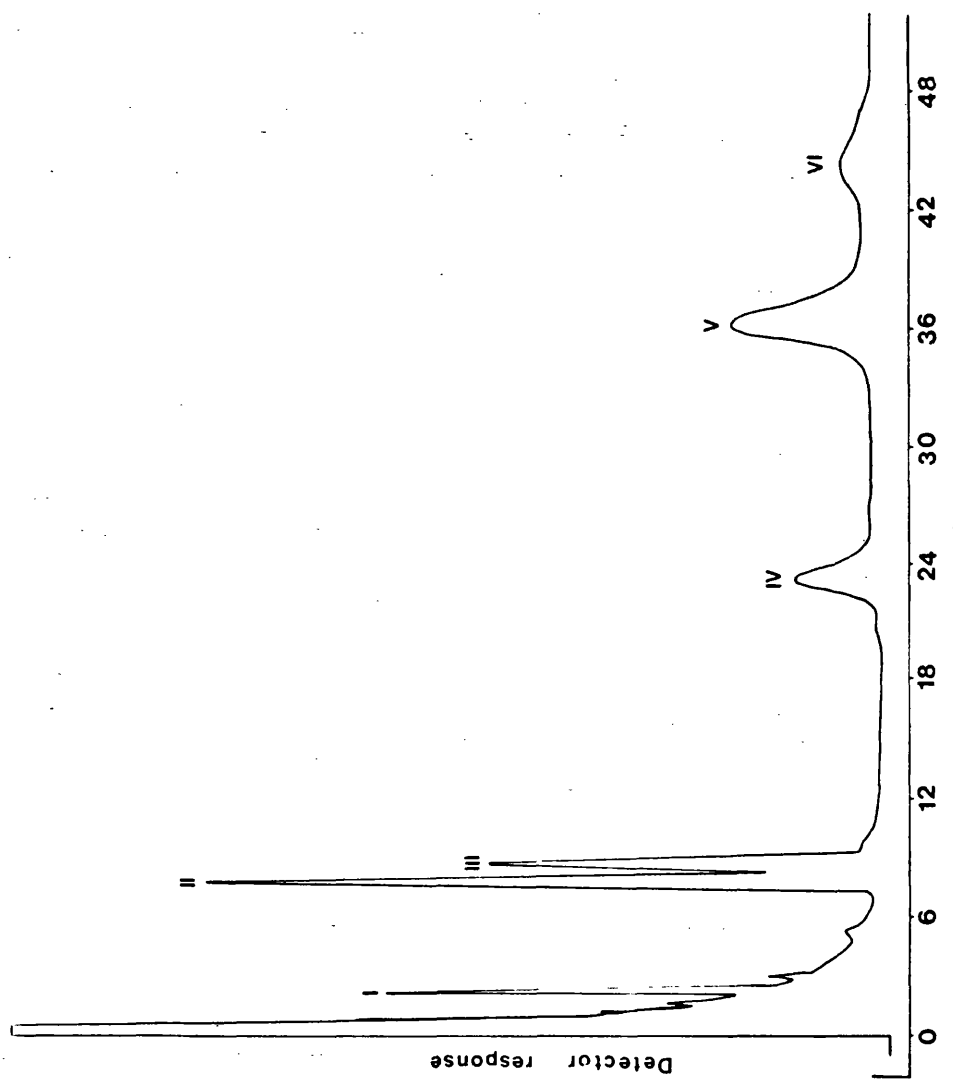


Fig. 42. Gas chromatogram of alditol acetates from hydrolysis of native SP glycopeptide. For identity of peaks I - VI and chromatography conditions see Fig. 32.

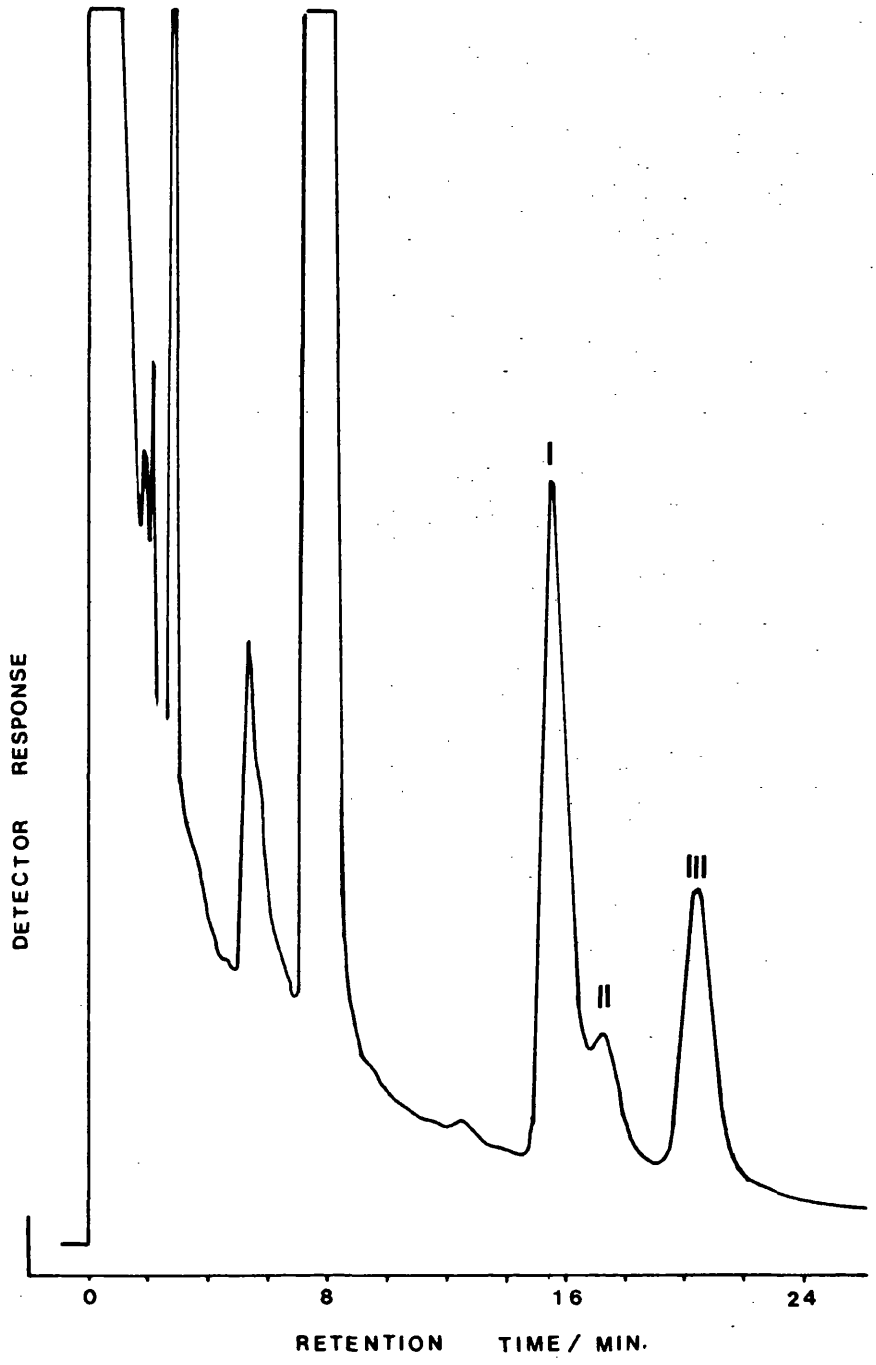


Fig. 43. Gas chromatogram of alditol acetates from hydrolysis of native SP glycopeptide. For identity of peaks I -III and chromatography conditions see Fig. 33.

II, and III in Fig. 43 were similarly identified as the alditol acetates of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and perseitol respectively. The relative average concentrations of the constituent sugars are shown in Table 8. Again, derivatives of the desialylated SP glycopeptides obtained by the same procedure gave the same pattern of sugars (apart from sialic acid) in the same relative proportions indicating that the hydrolysis step has no effect on the sugars other than sialic acid.

Periodate oxidation of the sialic acid-poor glycopeptides

Samples of the purified desialylated SP glycopeptides (10 mg) in 0.05 M sodium acetate buffer, pH 4.5, (2.5 ml) containing 50 mM sodium metaperiodate were treated with periodate as described for the SR glycopeptide. Samples (200 µg) of the periodate treated SP glycopeptide were hydrolysed with FTA and converted to alditol acetates as described for the native glycopeptide. The chromatographic trace obtained from injections onto the column of OV-225 is shown in Fig. 44 and that obtained in from injections onto the column of OV-17 is shown in Fig. 45. Peaks I, II and III in Fig. 44 were identified by comparison with authentic standards as the alditol acetates of D-mannose, persetiol and N-acetyl-D-glucosamine respectively. Peaks I and II in Fig. 45 were similarly identified as the alditol acetates of N-acetyl-D-glucosamine and perseitol respectively. Relative concentrations of the constituent sugars of theperiodate treated SP glycopeptide are shown in Table 8. Control experiments carried out by omitting the periodate step showed that the carbohydrate content of the glycopeptide was the same as that of the native sialoglycopeptide. As can be seen from Table 8, fucose, galactose and galactosamine were completely destroyed by periodate whereas approximately 44% of mannose was destroyed and

Source	Monosaccharide components expressed as μmol/100 mg peptide					
	L-fucose	D-mannose	D-galactose	N-acetyl-D-glucosamine	N-acetyl-D-galactosamine	N-acetyl neuraminic acid
Native sialoglycopeptide	18.9	50.16	33.33	42.98	13.12	14.18
Periodate treated sialoglycopeptide	trace	28.33	trace	38.91	trace	0.00
T. foetus treated sialoglycopeptide	3.11	7.77	6.11	11.49	6.15	0.00

Table 8. Composition of the amounts of individual monosaccharides present in the native, periodate and T. foetus treated sialic acid poor (SP) glycopeptide. Values expressed for hexoses and hexosamines were calculated from gas chromatographic data as described in the Materials and Methods section. N-acetyl neuraminic acid was estimated colorimetrically by the modified thiobarbituric acid of Aminoff (1961).

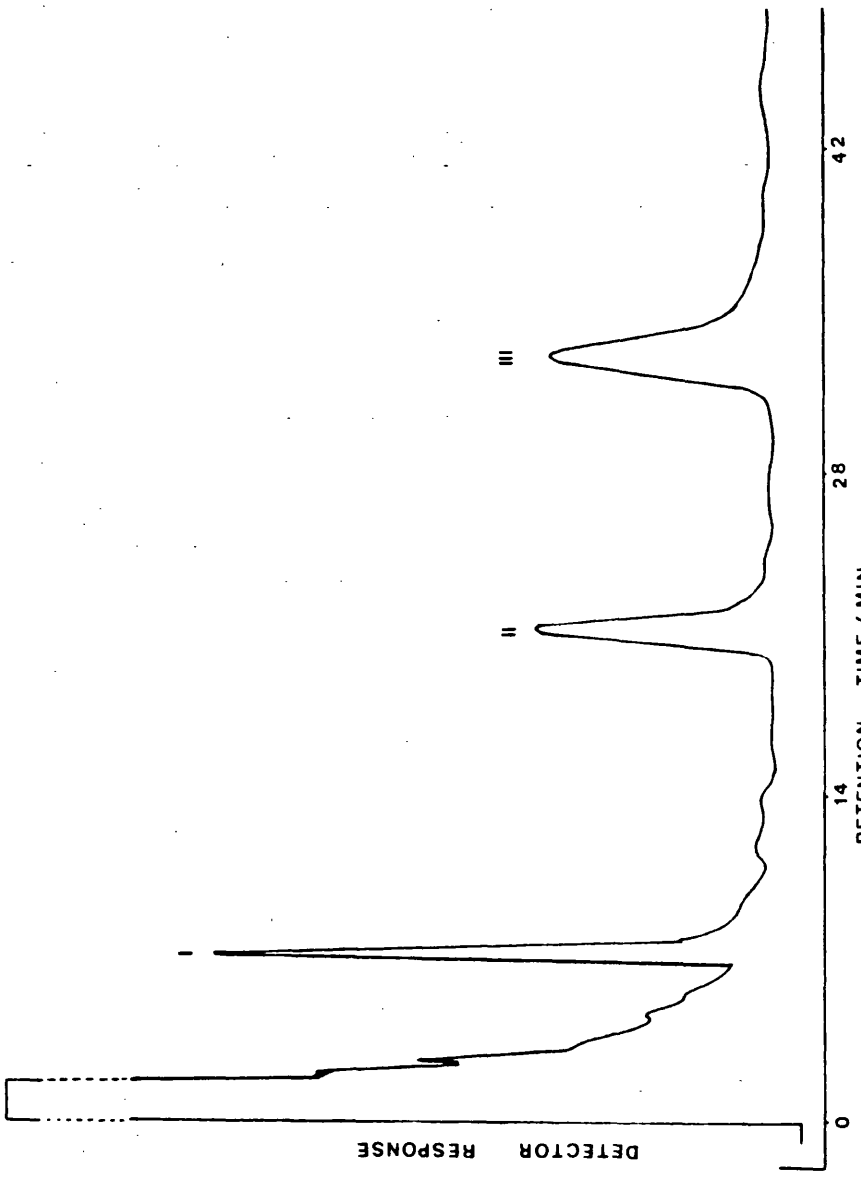


Fig. 44. Gas chromatogram of alditol acetates from hydrolysis of periodate treated SP glycopeptide. Peaks I -III correspond to: D-mannose, perseitol and N-acetyl-D-glucosamine respectively. For chromatography conditions see Fig. 32.

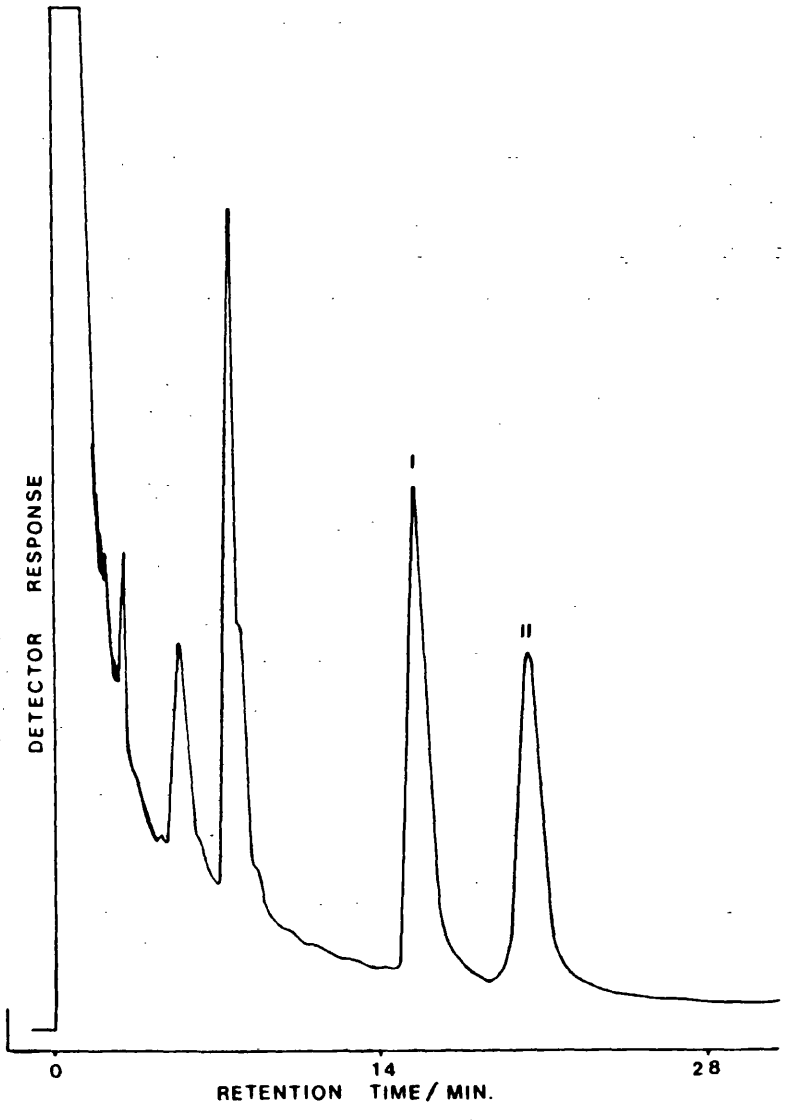


Fig. 45. Gas chromatogram of alditol acetates from hydrolysis of periodate treated SP glycopeptide. Peaks I and II represent N-acetyl-D-glucosamine and perseitol respectively. For chromatography conditions see Fig. 33.

glucosamine was totally protected from the action of periodate.

Treatment of the sialic acid-poor glycopeptides with mixed glycosidases from Trichomonas foetus

The purified desialylated SP glycopeptide (10 mg) was treated with T. foetus extract (10 mg) as described for the SR glycopeptide. The mixture was then passed through a column (100 x 1.5 cm) of Sephadex G-25. The column was eluted with 0.1 M acetic acid with a flow rate of 40 ml/h. Fractions (3 ml) were automatically collected and continuously monitored at 240 nm and aliquots (0.1 ml) were assayed for hexose. The elution profile obtained(Fig. 46) showed an extra peak (Peak II) in comparison with the elution profile of the T. foetus treated SR glycopeptide (Fig. 40). Fractions corresponding to the excluded peak (peak I, Fig. 46) and to peak II (Fig. 46) were pooled separately, freeze-dried and samples (200 µg) were converted to alditol acetates as described above. The chromatographic trace obtained from injections of derivatives of peak I (fig. 46) onto the column of OV-225 is shown in Fig. 47 in which peaks I, II, III, IV, V, VI and VII were identified as the alditol acetates of L-fucose, D-mannose, D-galactose, D-glucose, persetol, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. The chromatographic trace obtained from injections of derivatives of peak II (Fig. 46) onto the column of OV-225 showed that it is composed largely of D-glucose together with traces of D-mannose and D-galactose (Fig. 48) and was in fact the same as that obtained from alditol acetate derivatives of T. foetus extract itself. This suggests that peak II (Fig. 46) is in fact fragments of the enzyme and that peak I (Fig. 46) represents the residual SP glycopeptide.

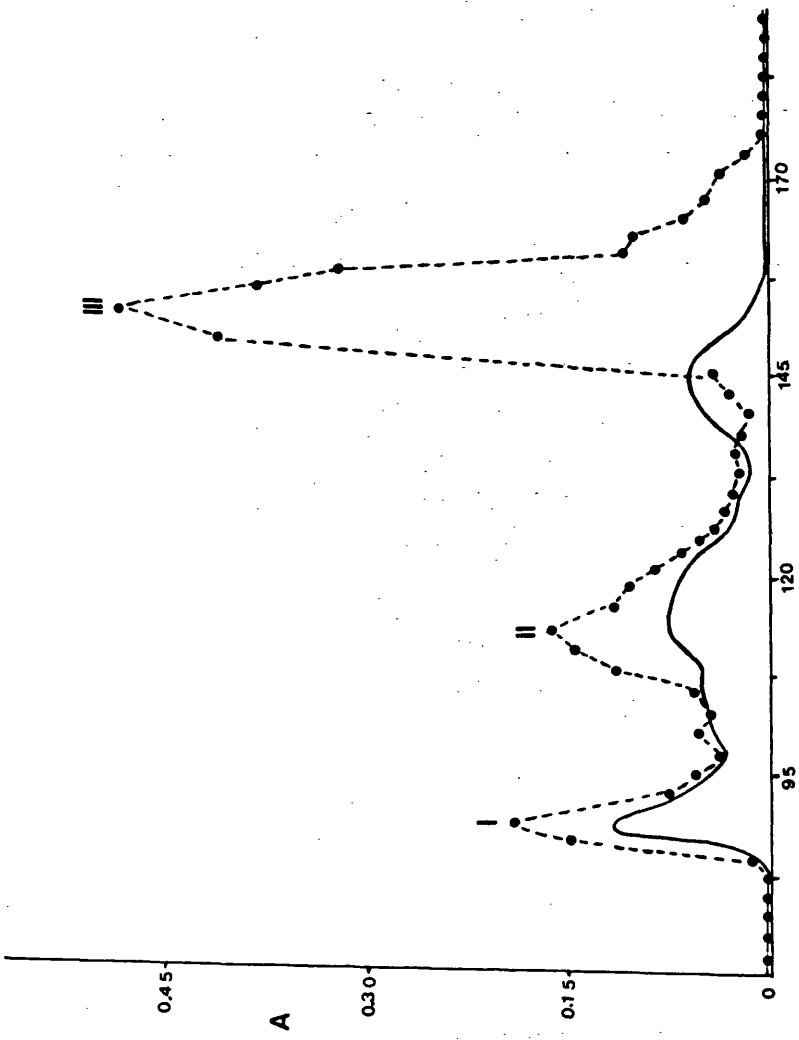


Fig. 46. Desalting on Sephad-x G-25 of T. foetus - treated SP glycopeptide. The column was eluted with 0.1 M acetic acid . Aliquots (0.1 ml) of fractions were individually assayed for hexose (A_{420} ●). Protein (A_{240} —) was continuously monitored using a Cecil 272 spectrophotometer.

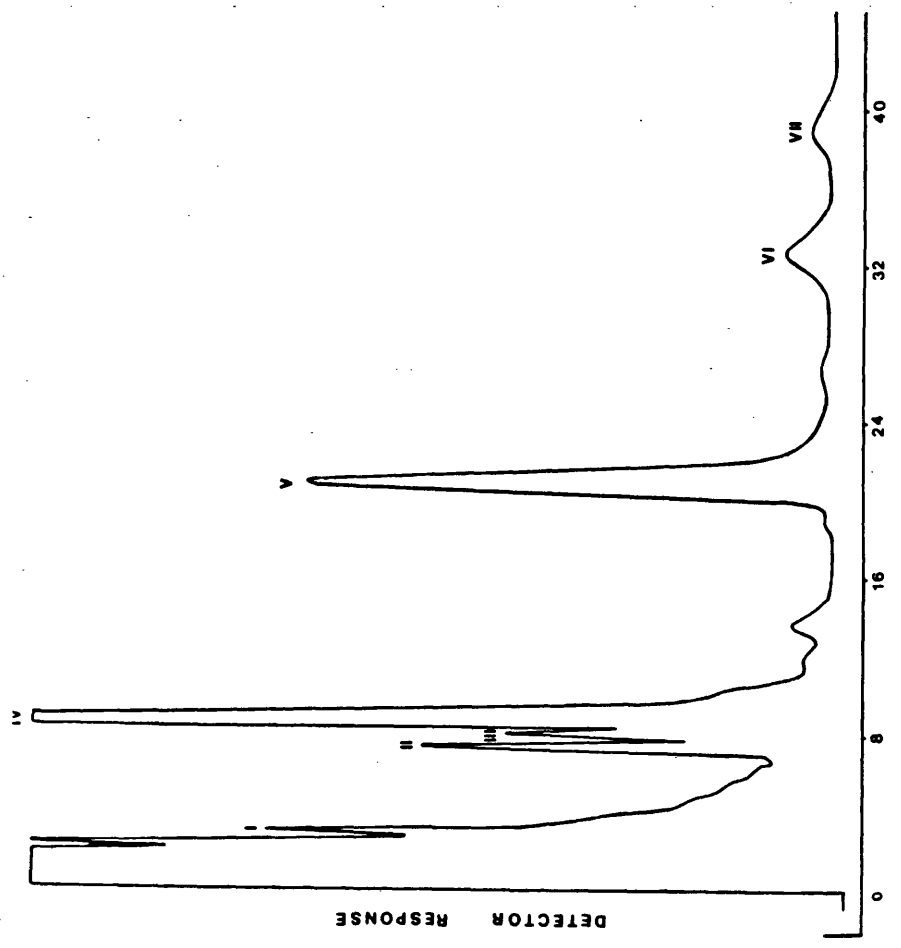


Fig. 47. Gas chromatogram of alditol acetates from hydrolysis of T. foetus - treated SP glycopeptide. For chromatography conditions and identity of peaks I - VII see Fig. 41.

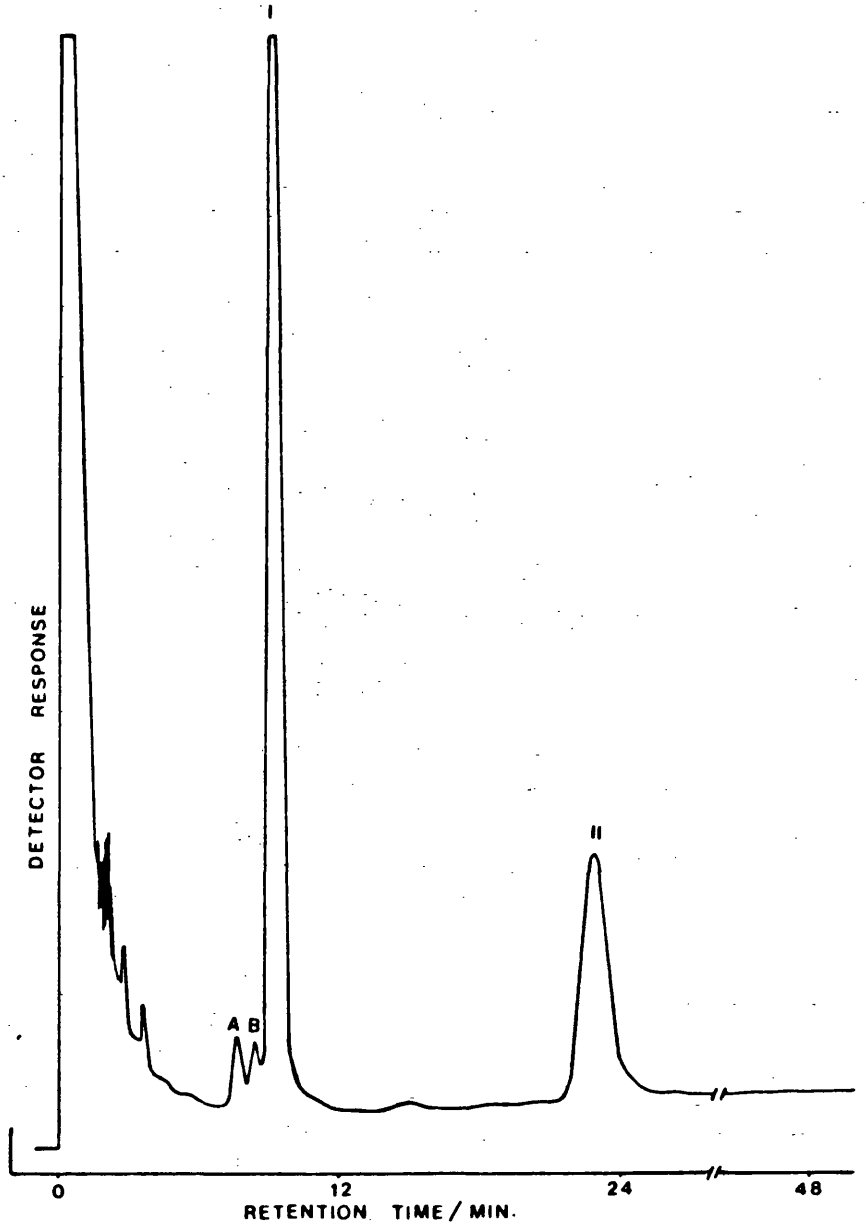


Fig. 48. Representative gas chromatograms of alditol acetates from hydrolysis of samples corresponding to peak II (Fig. 46) and also from hydrolysis of samples of T. foetus extract. A and B represent traces of D-mannose and D-galactose respectively. Peaks I and II represent D-glucose and perseitol respectively. For chromatography conditions see Fig. 32.

Control experiments performed by replacing the enzyme with buffer alone or with heat inactivated (100°C, 10 min) enzyme showed that the carbohydrate content of the glycopeptide was the same as that of the native glycopeptide. Relative concentrations of the constituent sugars of the T. foetus treated SP glycopeptide are shown in Table 8.

Amino acid analysis

The amino acid contents of the SR and SP glycopeptides were determined (Materials and Methods) by using a Chromaspek amino acid analyser. Samples (160 µg) were hydrolysed (110°C, 22h) with 3N-Mercaptoethanesulfonic acid (200 µl). The acid was neutralised and samples (200 µl) were injected directly onto the amino acid analyser. The data obtained are expressed as nmol/ml sample (Table 9).

The SR glycopeptide showed the presence of relatively high amounts of serine, threonine, glutamic acid, glycine and leucine in comparison with the SP glycopeptide which was found to contain relatively high amounts of aspartic acid. In both glycopeptides only traces of the aromatic amino acids (tyrosine and phenylalanine) were detected. This was expected as both fractions showed a very low absorbance at 280 nm.

Immunochemical Studies

I) Immunochemical characterization of the sialic acid-rich glycopeptide fraction

i) Generation of antisera

Antibody-containing serum against the purified SR glycopeptide

Amino acid	SR glycopeptide	SP glycopeptide
Aspartic acid	39.3	102.3
Threonine	103.0	40.3
Serine	131.1	52.1
Glutamic acid	31.6	9.6
Proline	ND	ND
Glycine	32.6	10.8
Alanine	68.8	38.3
Cystine	ND	ND
Valine	18.8	25.5
Methionine	6.4	trace
Isoleucine	2.4	trace
Norleucine	ND	ND
Leucine	17.8	trace
Tyrosine	trace	trace
Phenylalanine	trace	trace
Histidine	24.0	11.4
Lysine	6.6	6.0
Arginine	3.5	trace
Tryptophan	trace	trace

Table9. Amino acid composition of the SR and SP glycopeptides.

ND = not determined. Values are nmol/ml sample.

was raised in rabbits (Materials and Methods). Anti SR glycopeptide antibodies were first detected, by means of Ouchterlony double-diffusion experiments, approximately 8 weeks after the first injection of the rabbit and the highest antibody titre was detected approximately 14 weeks after the first injections.

ii) Ouchterlony Double-diffusion

Ouchterlony double-diffusion experiments in which anti SR glycopeptide antisera was tested against a serial dilution of the antigen showed that the end point, as judged by the absence of precipitin lines, was within the range of 2^{3-4} (titres are expressed as the reciprocal of dilution). The end points obtained with serial dilution of the desialylated glycopeptide antigen were within the range of 2^{5-6} suggesting that carbohydrate determinants could play a role in the antigenicity of the SR glycopeptide. Precipitates were also obtained with the crude (membrane material before partial purification on Sephadex G-50) pronase-digest and with the partially purified (PP) sialoglycopeptide off Sephadex G-50 (Fig. 25). In both cases the end points were within the range of 2^{1-2}. Only very faint diffuse precipitates were obtained using the SP glycopeptide indicating that the anti SR glycopeptide antisera is fairly specific for the SR glycopeptide. Neither the periodate-treated SR glycopeptide nor the T. foetus-treated SR glycopeptide were able to interact with the anti SR glycopeptide.

(iii) Agglutination of bovine milk fat globules

The ability of the anti SR glycopeptide antibodies to interact with pooled bovine milk fat globules was studied and, as

expected, the globules were found to be agglutinated by relatively high dilutions of the antibody (Table 10).

Inhibition studies showed that only the native and desialylated glycopeptides antigen were able to inhibit the agglutination of bovine milk fat globules by four agglutination doses of the antibody. As shown with the immunodiffusion experiments, neither the periodate treated SR glycopeptide nor the T. foetus treated SR glycopeptide were able to inhibit the agglutination of the bovine globules by four agglutination doses of the antibody (Table 11) indicating that carbohydrate residues are involved in the antibody binding site.

In view of the evidence, obtained from periodate and glycosidase treatment, of the importance of carbohydrate residues in the ability of SR glycopeptide to inhibit agglutination, the effect of various monosaccharides on the agglutination assay was investigated. Of the range of monosaccharides used in the inhibition assay only N-acetyl-D-galactosamine and to a lesser extent D-galactose were able to strongly inhibit the agglutination of the bovine globules by four agglutination doses of the antibody (Table 12). N-acetyl-D-glucosamine was found to be a weak inhibitor, whereas D-mannose and L-fucose did not show any inhibition effect (Table 12).

II Immunochemical characterisation of the sialic-acid-poor glycopeptide fraction

i) Generation of antisera

Antibody-containing serum against the purified SP glycopeptide fraction was raised in rabbits (Materials and Methods). Anti SP

Antiserum	Washed bovine milk fat globules
Anti SR glycopeptide antisera	2^{5-6}
Anti SP glycopeptide antisera	2^{5-6}
Anti whole MFGM antisera (raised in rabbits)	2^{7-8}
Anti whole MFGM antisera (raised in sheep)	2^{6-7}

Table 10. Agglutination titres of washed bovine milk fat globules against serial dilutions of antisera raised against MFGM and MFGM-derived sialoglycopeptides. Titres are expressed as the reciprocal of the minimum dilution of agglutinin (antisera) resulting in no agglutination of the globules suspension.

Inhibitor \ Agglutinin	Anti whole MFGM antisera	Anti SR glycopeptide antisera	Anti SP glycopeptide antisera
SR-glycopeptide	-ve	2.6 mg/ml	-ve
desialylated SR-glycopeptide	-ve	1.3 mg/ml	-ve
Periodate treated SR glycopeptide	-ve	-ve	-ve
T.foetus treated SR glycopeptide	-ve	-ve	-ve
SP-glycopeptide	2.6 - 1.3 mg/ml	-ve	0.66 - 0.33 mg/ml
Periodate treated SP glycopeptide	-ve	-ve	-ve
T. foetus treated SP glycopeptide	-ve	-ve	-ve
Sialoglycopeptide mixture, off Sephadex G-50	1.3 mg/ml	10.6 mg/ml	6.2 mg/ml

Table 11. The ability of native and modified MFGM-derived sialoglycopeptides to inhibit antisera-induced agglutination of washed bovine milk fat globules. Titres are expressed as the minimum concentration of the sialoglycopeptides in the final volume to inhibit, completely, the agglutination of the globules by four agglutination doses of the antisera.

Inhibitor concentration	L-fucose	D-mannose	D-galactose	N-acetyl-D-glucosamine	N-acetyl-D-galactosamine
1.0 M	++++	++++	-	++	-
0.5			+	++	-
0.25			++	++++	-
0.125			+++	++++	-
0.063			++++		++
0.031					+++
0.016					++++

Table 12. The ability of a range of monosaccharides to inhibit the anti SR glycopeptide antiserum-induced agglutination of washed bovine milk fat globules. Globule agglutination was scored as ++++, (x>90%), +++, (70% < x < 90%), ++, (50% < X < 70%), + (30% < X < 50%) and - (x < 30%). Controls without antisera were found to give agglutination up to 20%.

glycopeptide antibodies were first detected in relatively high titres (2^{3-4}) only four weeks after the first injection indicating that the SP glycopeptide is a stronger immunogen than the SR glycopeptide. The highest antibody titre (2^{6}) was detected approximately 10 weeks after the first injection.

ii) Ouchterlony double-diffusion

As with the case of SR glycopeptide, precipitin lines were observed when Anti SP glycopeptide antisera were tested against native SP glycopeptide, the crude (membrane material before partial purification on Sephadex G-50) and against the PP sialoglycopeptide (Fig. 25). Neither the periodate treated SP glycopeptides nor the T. foetus treated SP glycopeptide were apparently able to interact with the anti SP glycopeptide antisera. No interaction was obtained with the SR glycopeptide.

(iii) Agglutination of bovine milk fat globules

Again, pooled bovine milk fat globules were found to be agglutinated by relatively high dilutions of anti SP glycopeptide antisera (Table 10). Inhibition studies showed that the agglutination of the globules by four agglutination doses of the anti SP glycopeptide antisera was inhibited the native SP glycopeptide and by the PP sialoglycopeptide (Table 11). The periodate treated SP glycopeptide and the T.foetus treated SP glycopeptide showed no inhibitory effect (Table 11).

Inhibition studies using a range of monosaccharides showed that N-acetyl-D-gucosamine and to a lesser extent D-galactose were able to inhibit the agglutination of the globules by four agglutination

doses of anti SP glycopeptide antisera (Table 13).

III. Studies with anti whole bovine MFGM antisera

Anti whole bovine MFGM antisera was kindly provided by Dr. G.H. Farrar, University of Bath, with the information that it agglutinates bovine milk fat globules and interacts only with the PP sialoglycopeptide and SP glycopeptide.

Inhibition studies showed the same pattern observed with the case of anti SP glycopeptide antisera. Thus the SP glycopeptide and the PP sialoglycopeptide together with N-acetyl-D-glucosamine inhibited the globule's agglutination (Tables 11, 14). The inhibitory activity of the SP glycopeptide was completely destroyed by corresponding treatment with periodate or glycosidases (T. foetus).

Inhibitor Concentration	L-fucose	D-mannose	D-galactose	N-acetyl-D-glucosamine	N-acetyl-D-galactosamine
1.0 M	++++	++++	+	-	++++
0.5			+	+	
0.25			++	+	
0.125			++	++	
0.063			+++	+++	
0.031			++++	++++	
0.016					

Table 13. The ability of a range of monosaccharides to inhibit the anti SP glycopeptide antiserum induced agglutination of washed bovine milk fat globules. Globule agglutination was scored as ++++, (X > 90%), +++, (70 < X < 90%), ++, (50% < X < 70%), + (30% < X < 50%) and (X < 30%). Controls without antisera were found to give agglutinations of up to 20%.

Inhibitor Concentration	L-fucose	D-mannose	D-galactose	N-acetyl-D-glucosamine	N-acetyl-D-galactosamine
1.0 M	++++	++++	+++	-	++
0.5			++++	+	++
0.25				++	+++
0.125				+++	++++
0.063				++++	
0.031					
0.016					

Table 14. The ability of a range of monosaccharides to inhibit the anti whole MFGM antisera induced agglutination of washed bovine milk fat globules. Globule agglutination was scored as ++++, (X > 90%), +++, (70 < X < 90%), ++, (50% < X < 70%), + (30% < X < 50%), and (X < 30%). -

Controls without antisera were found to give agglutinations of up to 20%.

Discussion

In view of the mechanism of secretion of milk fat globules in which the emerging globule is coated by the apical membrane of the secretory cell, which then becomes the milk fat globule membrane (MFGM), the latter membrane can serve as a convenient source and model for epithelial mammalian membranes in general (General Introduction to this thesis). The glycoproteins of the membrane surface, which extend out onto the aqueous surrounding are of particular interest in that they might be expected to carry many of the recognition characters of the parent membrane and the present thesis is largely concerned with an examination of the structure, exposure and function of these glycoproteins.

Most previous work on the glycoprotein components of bovine MFGM has concentrated on the isolation and characterisation of the intact molecules extracted in various ways from the membrane. Membrane proteins obtained in this way are generally hydrophobic and can only be maintained in solution throughout the isolation and fractionation procedures by the use of detergents. In the present studies, proteolytic enzymes are used to cleave the exposed glycopeptides from the outer surface of intact milk fat globule membrane. In this way water soluble glycopeptides are obtained which should contain many of the recognition factors of the original membrane.

Prior to their treatment with proteases, it is necessary to wash the fat globules with water or isotonic buffer in order to remove entrained or adsorbed milk serum components (e.g. casein) (Patton and Keenan, 1975). Such washing may well also remove loosely associated membrane constituents and while this is a disadvantage, it is one that

is common to nearly all membrane fractionation procedures. Treatment of washed bovine milk fat globules with pronase resulted in the release of a sialoglycopeptide fraction which was chromatographed on Sephadex G-50 as a single peak containing approximately 80 - 90% of the sialic acid and 60% of the hexose applied to the column. Control experiments using milk fat globules in the absence of pronase and vice versa showed that the glycopeptide fraction was indeed proteolytically cleaved from the globule surface. Further fractionation of the sialoglycopeptide fraction on a column of DEAE-Sepharose CL 6B gave two sialoglycopeptide fractions, the sialic-acid-poor (SP) and the sialic acid-rich (SR) glycopeptide. The SP and SR glycopeptides were further analysed by gel filtration on Sephadex G-75 which showed that both glycopeptides were homogenous (in terms of size) and that their respective molecular weights were 13,000 and 23,000.

The molecular weights of the sialoglycopeptides and their homogeneity were further investigated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). The method employed for preparation of acrylamide gels was that described by Weber et al.(1972). However, when staining was carried out according to this method it was not possible to visualise any bands for either the SP or the SR glycopeptide in spite of the fact that bands corresponding to the protein standards were easily obtained. The absence of protein bands in the case of SP and SR glycopeptides could probably be due to the fact that fixing (by methanol-glacial acetic acid) of the glycopeptides was not effective and they were consequently washed away. Recently, Vesterberg et al. (1977) described a procedure for fixing proteins in isoelectric

focusing gels which involves the use of trichloroacetic acid (TCA) in the presence of sulphosalycilic acid. Fixing the gels according to this method and subsequent staining and destaining as described by Weber et al.(1972) allowed the detection of protein bands corresponding to the SP and SR glycopeptides, although they were relatively faint in the case of the former.

Glycoproteins are known to behave anomalously in molecular weight determinations on SDS-PAGE and an empirical procedure has been developed to take account of this (Sergrest et al., 1971). Apparent molecular weights of glycoproteins, determined from mol. wt./mobility plots of standard proteins, are found to decrease with increasing acrylamide gel concentration, asymptotically approaching a limiting value. Extrapolation of such a curve to the asymptotic minimum value has accordingly been recommended for molecular weight determinations of glycoproteins. Application of this procedure to the SP and SR glycopeptides gave molecular weights of 17,000 and 23,000 respectively (Fig. 30). While molecular weight estimation of the SR glycopeptide by SDS-PAGE was the same as that obtained by gel filtration, the former technique gave a higher value in the case of SP glycopeptide. It may well be that the SDS-PAGE value is too high in the case of the SP glycopeptide. It is generally acknowledged that glycoproteins binds less SDS than normal proteins and so migrate less far. This descrepancy could be at least partially compensated in the SR glycopeptide which has a high content of negative-charged sialic acid.

The Sp glycopeptide fraction was found to contain relatively high levels of D-mannose and N-acetyl-D-Glucosamine together with

lower levels of L-fucose, D-galactose, N-acetyl-D-galactosamine and sialic acid. The presence of high levels of mannose and glucosamine in this fraction suggests that the majority of the oligosaccharide chains of this glycopeptide are N-glycosidically linked to asparagine which was indeed found to be the major amino acid on the SP glycopeptide. Thus, the major oligosaccharide chains of the SP glycopeptide could be of the 'simple' - type (Fig. 12) which contain only D-mannose and N-acetyl-D-glucosamine and/or of the 'complex'-type which additionally contains galactose and sialic acid. (Fig. 12.). The structure shown in Fig.12 has been suggested (Montreuil, 1975) as being typical of N-glycosically linked oligosaccharides of soluble glycoproteins and the limited studies that have been carried out on membrane-bound glycoproteins suggest that it may well apply to them also (Sturgess et al, 1978; Kornfeld and Kornfeld, 1980). The SR glycopeptide, on the other hand, was found to contain relatively high levels of D-galactose, N-acetyl-D-galactosamine and sialic acid together with lower levels of D-mannose and N-acetyl-D-glucosamine. The presence of high levels of galactose and galactosamine in the SR glycopeptide suggests that the majority of the oligosaccharide chains in this fraction are O-glycosidically linked to serine and/or threonine which were indeed the major amino acids in this glycopeptide fraction (see later).

Treatment of the SR glycopeptide with alkaline borohydride resulted in the release of a tetrasaccharide which has previously (Farrar, 1978) been established as N-acetylneuraminyl(2→3)-β-D-galactopyranosyl-(1→3)-[N-acetylneuraminyl-(2→6)] -N-acetyl-D-galactosaminitol. In addition to this tetrasaccharide, Farrar (1978)

reported the isolation (from the equivalent of peak II in Fig. 34) of two trisaccharides (N-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-N-acetylgalactosaminitol and β-D-galactopyranosyl-(1→3)-N-acetylneuraminyl-(2→6)-N-acetyl-D-galactosamininitol. In all my experiments the tetrasaccharide was always recovered intact and with the same reported composition. Material corresponding to peak II (Fig. 34), on the other hand, was found to show a composition corresponding to that of a mixture of the trisaccharides contaminated by relatively high levels of protein from the residual glycopeptide. Farrar (1978) showed that the trisaccharide mixture can be recovered in relatively pureform by thin layer chromatography or by high voltage electrophoresis and paper chromatography (Lisowaska et al., 1980). The protein contamination of the released oligosaccharides is probably due to reductive degradation of peptide bonds by sodium borohydride. The ability of sodium borohydride to induce reductive degradation was utilized to isolate Fc fragments from IgM, IgG (Yakulis et al., 1968) and IgA (Yakulis et al., 1969). These studies showed that sodium borohydride acts firstly to reduce the sulfhydryl bonds linking the subunits of these immunoglobulins and then on the peptide bonds between Fc and Fab fragments. It is of interest to mention that in the case of IgM only the bond linking the Fab and Fc appears to be affected by sodium borohydride and that it is conceivable that sodium borohydridge alters the special configuration of the protein in a way to make this area of the chain available for reductive cleavage without affecting the other ones (Yakulis et al. 1968). Protein degradation was also reported by Lisowska (1969) who showed that alkali-labile oligosaccharides isolated from M and N active glycoproteins and glycopeptides were associated (but not

linked) with low molecular weight peptides that were liberated during their treatment with alkaline borohydride. Again this possibility was not ruled out by Iyer and Carlson (1971) and Lisowska et al., (1980). Protein degradation of the SR glycopeptide following its treatment with alkaline borohydride was particularly reflected in the low yield of the residual (alkali-stable) glycopeptide.

Periodate treatment of the SP glycopeptide resulted in the complete destruction of fucose, galactose and N-acetylgalactosamine whereas approximately 44% of mannose was destroyed while N-acetylglucosamine was protected. This suggests that the C_3 and/or C_4 positions in galactose and N-acetylgalactosamine are free and approximately 50% of these positions are free in mannose. Fucose is usually terminal and its complete destruction by periodate was expected. Similar treatment of the SR glycopeptide resulted in the destruction of approximately 30% of galactose and of mannose whereas N-acetylgalactosamine and N-acetylglucosamine were protected. These results suggest that approximately 70%of the galactose and of the mannose residues are substituted at position C_3. As with the SP glycopeptide, fucose was completely destroyed by periodate suggesting that it is terminal in the oligosaccharide chains.

Treatment of the desialylated SR glycopeptide with T. foetus extract resulted in the release of approximately 80% of the original galactose and 56% of the original N-acetylgalactosamine. The total carbohydrate release was approximately 42% (apart from sialic acid). Fucose, mannose and N-acetylglucosamine were protected from the action of T. foetus glycosidases. This could have resulted from the

presence on the SR glycopeptide oligosaccharide chains of a terminal sugar (possibly fucose) which is joined to the penultimate sugar by a linkage which is resistant to the action of the corresponding enzyme (fucosidase) in the T. foetus extract as suggested by Westwood et al.(1976). Thus, in such cases there will be intact sequences of monosaccharides from the N-acetylglucosamine linked to the asparagine through to the terminal sugar (fucose). Similar treatment of the desialylated SP glycopeptide, but by using double the T. foetus extract concentration, resulted in the release of approximately 78% of the original carbohydrates (apart from sialic acid). This is consistent with the results of Westwood et al.(1976) who showed that treatment of the carcinoemberyonic antigen (CEA) with T. foetus extract resulted in the release of 70 - 80% of the CEA carbohydrates, but there was no report on the concentration of T. foetus extract used in their experiment. The release of approximatly 84% of fucose, 85% of mannose, 73% of N-acetylglucosamine and 53% of N-acetylgalactosamine as a result of treatment of the desialylated SP glycopeptide with T. foetus glycosidases suggests that terminal fucose resistant residues, if present, are less in the SP glycopeptide than in the SR glycopeptide although it must be borne in mind that the concentration of the T. foetus extract with the former was double that used with the latter. It is important,in the context of the immunological studies to mention that the T. foetus glycosidases have been shown to have no proteolytic activity (Watkins, 1966a; Westwood et al., 1976). Further studies are required to establish the optimal conditions to achieve maximum carbohydrate release.

Preliminary amino acid analyses of the SP and SR glycopeptides

were carried out by hydrolysis of the glycopeptides with 3M-mercaptoethanesulphonic acid (105°C, 22h). The results showed a clear difference in the amino acid composition of the two glycopeptides. The SR glycopeptide was found to contain relatively high levels of serine, threonine, glutamate and glycine in comparison with the SP glycopeptide which was found to contain relatively high levels of aspartate (asparagine). The high levels of asparagine and low levels of serine and threonine in the SP glycopeptide could indicate that the majority of the oligosaccharide chains of the SP glycopeptide are most likely to be of the N-glycosidically linked type. This is consistent with the fact that the major carbohydrates of the SP glycopeptide are mannose and N-acetylglucosamine. Similarly high levels of serine and threonine together with low levels of asparagine could indicate that the majority of the oligosaccharide chains are most likelty to be of the N-glycosidically linked type. Again, this is consistent with the fact that the major carbohydrates of the SR glycopeptide are galactose and N-acetylgalactosamine. Hydrolysis for 36 and 48 hrs were not carried out because of technical problems with the amino acid analyser in the department.

In view of the presence of compelx glycoprotein and glycolipid molecules at the outer surface of animal cells, it might be expected that these molecules are involved in the interaction of cells with their surroundings (see the General Introduction, p. 24). It is an attractive concept that carbohydrate patterns might play a major role in cell surface recognition phenomena. In a number of cases this has been found to be so, while in others the contribution of carbohydrate to the demonstrated specificity of certain membrane

glycoproteins remains unclear. Taking this into consideration, one aim of this work was to study the possible contribution of surface carbohydrates of the bovine MFGM to the overall antigenicity of the membrane.

Antisera were raised in rabbits against both the SP and SR glycopeptides. The antibody response to the SP glycopeptide fraction was observed four weeks after the first injection of the rabbit whereas the antibody response to the SR glycopeptide was not detected until approximately 14 weeks after the first injection. The relatively late antibody response of the rabbit, in the latter case, could be due to the presence of high number of sialic acid residues in the glycopeptde antigen. This could mask the glycopeptide antigenic determinants (cryptic antigens). Alternatively the presence of a high negative charge on the glycopeptide molecules might limit the number of B cells which can bind to the antigen. These observations together with the fact that only the SP glycopeptide fraction interacted with anti whole MFGM antisera suggests that the major membrane antigens are carried on the SP glycopeptide and none of these antigens are carried on the SR glycopeptide or if it does they are masked (e.g. by sialic acid).

Pooled bovine milk fat globules were found to be agglutinated by anti whole MFGM antisera, anti SP glycopeptide antisera and anti SR glycopeptide antisera. The agglutination of the bovine globules induced by anti SP glycopeptide and by anti whole MFGM antisera was specifically inhibited by the SP glycopeptide and the partially purified (PP) sialoglycopeptide mixture off Sephadex G-50. The agglutination of the globules by anti SR glycopeptide

antisera was specifically inhibited by the native and desialylated SR glycopeptide and to a much lesser extent by the PP sialoglycopeptide mixture. The SR glycopeptide did not inhibit the agglutination of the globules by anti SP glycopeptide antisera and vice versa indicating that each antisera binds to different receptors on the membrane surface.

Inhibition studies using a range of monosaccharides showed that N-acetylglucosamine specifically inhibited the agglutination of the bovine globules by anti whole MFGM antisera and by anti SP glycopeptide antisera. On the other hand, N-acetylgalactosamine and to a lesser extent galactose were found to inhibit the agglutination of the globules by anti SR glycopeptide antisera. Thus,it seems that the antigenic determinants of the SP glycopeptide are associated with the alkali-stable (N-glycosidically linked) oligosaccharide chains whereas those of the SR glycopeptide are associated with the alkali-labile (O-glycosidically linked) oligosaccharide chains. The ability of N-acetylglucosamine to inhibit the agglutination of the bovine globules by anti whole MFGM antisera and by anti SP glycopeptide antisera and its failure to inhibit the agglutination of the globules by anti SR glycopeptide antisera supports the earlier conclusion that the major antigenic determinants on the bovine MFGM are carried on the SP glycopeptide fraction. The involvement of carbohydrate in the antigenic determinants of the bovine MFGM was further demonstrated by inhibition studies by using periodate treated and T. foetus extract treated glycopeptides. Treatment of the desialylated SR glycopeptide with T.foetus extract resulted in the release of approximately 80% of galactose and 56% of N-acetyl-

galactosamine. These losses in galactose and N-acetylgalactosamine could account for the inability of the T. foetus extract treated SR glycopeptide to inhibit the agglutination of the globules by anti SR glycopeptide antisera. Similarly the inability of the periodate treated SR glycopeptide to inhibit the globules agglutination by anti SR glycopeptide antisera could be attributed to the loss of 30% of galactose. Similarly, treatment of the SP glycopeptide with periodate or T. foetus extract rendered the glycopeptide unable to inhibit the agglutination of the globules by anti whole MFGM and by anti SP glycopeptide antisera.

The results obtained from the agglutination-inhibition assays were confirmed by immunodiffusion studies. Ouchterlony double diffusion experiments showed that anti SR glycopeptide antisera reacted only with native and desialylated SR glycopeptide and to a lesser extent with the PP sialoglycopeptide mixture off Sephadex G-50 as judged by the formation of precipitin lines. No interaction was observed between anti SR glycopeptide antisera and SP glycopeptide and vice versa suggesting that each antisera is specific to its antigen, i.e. the SR and SP glycopeptide has no common antigenic determinants. As shown by the agglutination-inhibition assays, T. foetus extract treated and periodate treated SR glycopeptide did not interact with anti SR glycopeptide antisera as judged by the absence of precipitin lines which again stresses the involvement of the carbohydrate residues in the antibody binding sites. This could be as a result of direct involvement of the carbohydrate residues in the antibody binding site, as judged by the agglutination-inhibition assays, or that the carbohydrate residues are very close

to the antigenic determinants on the glycopeptide antigen. As with the SR glycopeptide, T. foetus extract treated and periodate treated SP glycopeptide did not interact with either anti whole MFGM antisera and anti SP glycopeptide antisera.

Unlike the SR glycopeptide, the SP glycopeptide was able to form precipitin lines when tested against anti whole MFGM antisera which, again, suggests that the major antigenic determinants of the bovine MFGM are carried on the SP glycopeptide fraction.

Section B

Lectin receptors on the surface of milk fat globules.

Introduction

In view of the possible involvement of cell surface carbohydrate residues in a range of recognition phenomena (see the General Introduction) there is a need for reagents capable of detecting specific carbohydrate residues on the surface of intact cells. Lectins (also referred to as agglutinins, phytohaemagglutinins, phytoagglutinins and protectins) are a class of naturally-occurring compounds that go some way towards fufilling this requirement.

The main characteristics of lectins are their ability to bind sugars, to agglutinate cells, and to stimulate lymphocytes. The two latter activities depend upon the former and each lectin shows specificity in its ability to bind certain mono or simple oligosaccharides (Table 15).

Through their sugar-combining sites, lectins interact directly with polysaccharides and glycoproteins to form precipitates in a manner similar to the interaction of antibody with antigen, in that it is specific, exhibits concentration dependence on both lectin and polysaccharide or glycoprotein and may be inhibited specifically by low molecular weight 'haptens'-compounds identical with or derived from the sugar(s) for which the lectin is specific.

The occurrence, in plant extracts, of proteins that possess the ability to agglutinate erythrocytes was first reported by Stilmark back in 1888. This was followed by the introduction of lectins in immunological studies by Ehrlich in the early 1890's, who showed that specific immunity to the toxic lectins ricin (Ricinus communis)

Source	Carbohydrate* specificity
Plant	
Phytohaemagglutinin (red kidney bean)	D-GalNAc
Wheat germ agglutinin	D-GlcNAc, sialic acid
Ricinus communis (castor bean)	β-D-Gal
Soya bean agglutinin	D-GalNAc, D-Gal
Concanavalin A (jack bean)	α-D-Man, α-D-Glc
Lens culinaris (lentil)	Man, Glc
Arachis hypogaea (peanut)	TF-antigen, Gal
Bacterial	
Escherichia coli	Man
Salmonella typhimurium	Man
Pseudomonas aeruginosa	Gal
Animal	
Rabbit liver	Gal
Avian liver	GlcNAc
Helix pomatia (garden snail)	GalNAc
Limulus polyphemus (horseshoe crab)	NANA

Table 15. Some plant and animal lectins

* GalNac: N-acetylgalactosamine; GlcNAc; N-acetylglucoseamine; Gal: galactose; Man: mannose; Glc: glucose; NANA: N-acetyl-neuraminic acid.

and abrin (Abrus precatorius) could be achieved by repeated injection of small amounts of these antigens into white mice. In 1908, Landsteiner and Raubitschek established that the relative haemagglutinating activities of various seed extracts were quite different when tested with blood cells from different animals. In spite of this demonstration of species specificity, it was presumed for several decades that plant agglutinins were non-specific. It was not until the end of the 1940's that Boyd and Reguera (1949) and Renkonen (1948) indpendently discovered that certain seeds contain agglutinins specific for some human blood group antigens. The first evidence that sugars are determinants of blood group specificity was obtained in 1952 by Watkins and Morgan who studied the A-specific lectins and type H(O)-specific lectins from the serum of the eeel (Anguilla anguilla) and the seeds of Lotus tetragonolobus respectively. As haemagglutination by A-specific lectins was specifically inhibited by N-acetylgalactosamine, Watkins and Morgan (1952) concluded that this sugar serves as determinants of human blood group A specificity. Similarly, haemagglutination by type H(O)-specific lectins was specifically inhibited by methyl-α-L-fucopyranoside indicating that the α-L-fucopyranoside is the determinant of H(O) specificity. Both conclusions have been fully substantiated in subsequent studies (for reviews, see Watkins, 1972; Hakomori and Kobata, 1974).

In spite of the findings of Watkins and Morgan (1952), interest in lectins was limited until the discovery of the mitogenic activity of phytohaemagglutinin (Nowell, 1960) and of the preferential agglutination of malignantly transformed cells by wheat germ agglutinin (Aub et al., 1963, 1965; Burger and Goldberg, 1967).

As a result, lectin research and application entered, within a short period of time, a new and very active phase of development.

The nature of the cellular structures with which lectins interact has been probed by:

(i) Light- and electron-microscopic analysis of tissue sections following reaction with appropriately derivatized lectins (e.g. radiolabelled lectins, or lectins labelled with fluorescein, ferritin, Perioxidase or haemocyanin) [Collard et al., 1975; Friberg and Hammarstrom, 1975; Horisberger et al., 1977; Lis and Sharon, 1977].

(ii) Mono- and oligosaccharide inhibition of lectins or lectin derivatives (Lis and Sharon, 1977; Goldstein and Hayes, 1978).

(iii) Competitive binding between lectins of known specificity (Feller, et al., 1979)

(iv) The effect of enzymic or chemical modification on lectin reactivity (Novogrodsky et al., 1975; Carter and Sharon, 1976; Verbert et al., 1976; Lis and Sharon, 1977).

(v) Isolation of the reactive structures (Sharon and Lis, 1975; Lis and Sharon, 1977; Newman and Uhlenbruck, 1977; Goldstein et al., 1978; Lotan and Nicolson, 1979).

However, in applying any of these methods one should take great care that the lectins (or their derivatives) are homogenous and have been isolated with their biological activity unchanged. Moreover, their binding should be shown to be indeed sugar specific, i.e., it can be inhibited or reversed by the appropriate saccharide(s).

As a result of these studies many normal and transformed cells showed differential agglutinability when tested with concanavalin A (Con A) [Inbar and Sachs, 1969a;b; Moscana, 1971; Roth et al., 1975], wheat germ agglutinin (WGA) [Burger, 1969, 1973; Roth et al., 1975], Ricinus communis agglutinin (RCA) [Nicolson and Blautein, 1972; Roth et al., 1975], soya bean agglutinin (SBA) [Sela et al., 1970] and lentil lectin [Roth et al., 1975].

It was initially thought that the increased agglutinability of transformed cells resulted from the presence of a greater number of lectin binding sites on the latter cells (Lis and Sharon, 1977). This idea was supported by the fact that many untransformed cells which were not agglutinated by low concentrations of a lectin were agglutinated after mild proteolysis or neuraminidase treatment (Lis and Sharon, 1977). This suggested that cryptic (hidden) sites on the cell surface were being exposed by enzymic treatment resulting in agglutination. However, when the actual numbers of lectin on transformed (agglutinated) and their untransformed (non-agglutinated) counterparts were systematically compared it was found that with some exceptions [e.g. agglutination of erythrocytes and lymphocytes, by peanut lectin (Carter and Sharon, 1976; Novogrodsky et al., 1975)] there were no quantitative differences in binding of a given lectin to the two cell types. It has become clear that the reasons for the increased agglutinability of transformed cells are far more complex and that difference in this respect are also found between adult and embryonic cells and between interphase and mitotic cells (Stobo, 1972; Stobo et al., 1972; Mosier, 1974; Reisner et al., 1976 ; Sharon, 1976). Early ideas that high agglutinability could

also be directly connected with loss of contact inhibition (Burger, 1971) and tumorigenicity (de Micco and Berebbi,1972) have also been abandoned (Berman, 1975; Glimelius et al., 1975; Ukena et al., 1976) and many factors relating to both the cell surface and to the relevant lectin have been implicated in what is clearly a far from simple process (Table 16).

Another important effect of the interaction of lectins with cells is the triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation (Ling and Kay, 1975; Rosenthal, 1975; Openheim and Rosenstreich, 1976). As has been mentioned above, studies on lymphocyte activation began with the discovery by Nowell (1960) that phytohaemagglutinin (PHA) can stimulated lymphocytes to grow and divide. For a while PHA was the only mitogen known. This was followed by the discovery of two more mitogens both of which were lectins pokeweed mitogen (PWM) (Farnes et al., 1964) and the lectin from Wistaria floribunda (Barker and Farnes, 1967) . The most significant finding, in this field,was the discovery of the mitogenic effects of Con A (Wecksler et al., 1968; Douglas et al., 1969 ; Powell and Leon, 1970; Novogrodsky and Katchalski, 1971) because of the fact that Con A was the first mitogenic lectin whose activity could be readily inhibited in a reversible manner by low concentrations of simple sugars.

In earlier studies, mitogenic stimulation was estimated by counting under the microscope the number of lymphocytes that had been transformed into large, blast-like cells as a result of prolonged incubation (48 hours) with the mitogen tested. Now,

I. Properties of lectins:

(i) Number of saccharide-binding sites.

(ii) Strength of saccharide binding.

(iii) Electric charge.

(iv) Molecular size.

II. Chemical structures of cell surface receptors.

III. Cell surface properties:

(i) Number, accessibility and distribution of receptor sites.

(ii) Mobility of sites (membrane fluidity).

(iii) Electrical charge.

(iv) Surface rigidity.

(v) Surface structures (microvilli, etc.).

IV. Cytoplasmic components:

(i) Membrane peripheral proteins.

(ii) Microtubule-microfilament system.

(iii) Energy supply.

Table 16. Factors affecting cell agglutination by lectins.

(After Lis and Sharon, 1977).

mitogenic stimulation is determined by measuring the increase in the rate of incorporation of labelled thymidine, uridine or leucine into DNA, RNA or protein, respectively, after suitable periods of incubation of lymphocytes with the mitogen tested (Hirschhorn et al., 1974). More recently, however, Udey et al.,(1980) reported that WGA (a non mitogenic or anti-mitogenic lectin) and Con A (a well known mitogen) showed the same qualitative and quantitative effects on human peripheral blood mononuclear cells and purified lymphocytes in regard to at least three paraemters (protein phosphorylation, aminobutyric acid transport and biosynthetic labelling of particulate proteins) of early activation. They suggested that the absence of more marked DNA synthetic and complete proliferative responses in human lymphocytes stimulated with WGA may be due to delayed inhibitory or cytotoxic effects of WGA on monocytes and perhaps subpopulations of lymphocytes as well. These results emphasise the importance of studying more markers of activation other than the traditional procedures. Indeed, what is needed is an assay based on an early irreversible step in the activation process which in addition to being practical, rapid and precise, would provide a better estimate of the number of cells responding to an activator and the degree of activity of these cells (Waithe and Hirschhorn, 1978).

The observations of Bain et al.(1964) and of Bach and Hirschhorn (1964) that mixtures of lymphocytes from two genetically non-identical individuals are capable of stimulating each other in vitro (the mixed lymphocyte reaction), and the subsequent genetic and immunological analysis of this phenomenon (Bach and Amos, 1967; Albertini and Bach, 1968; Amos and Bach, 1968) established the mixed lymphocyte culture as a means of measuring the degree of histocompatibility

(see the General Introduction) between individuals and has provided an important tool for the study of immunobiology and genetics of tissue transplantation (Silvers et al., 1967; Hirschhorn, 1968; Schellekens et al., 1970; Dausset et al., 1970).

The major cell types responding to a stimulant are T lymphocytes and in vitro lymphocyte activation (transformation) is thought to reflect T cell function and cell-mediated immunity (CMI) (Bach et al., 1971; Greaves and Janossy, 1972). Non-specific transformation by mitogens can be used as an indicator of overall T cell function since a high percentage of cells are 'triggered' normally and a low level of transformation usually correlates well with depressed CMI. A significant degree of transformation (i.e. at least three times greater than the spontaneous incorporation) to a given antigen usually indicates prior sensitivity to that antigen (Urbaniak et al., 1978).

Transformation tests are technically demanding and are subject to many variables. It is essential to include a normal control or even better a 'reference pool of responders' with each experiment. If responses are significantly different from control values serial tests are necessary for confirmation. Some patients (e.g. cancer-bearing subjects) may have inhibitors present in their serum which will be detected if cultures are done with autologous as well as with 'standard' sera. Conversely, if cultures are always performed with autologous serum some negative results may be due to the presence of inhibitors and not to depression of CMI (Whittaker et al., 1971; Knocke et al., 1974). Methods of lymphocyte stimulation have been

reviewed by a number of authors (Oppenheim and Rosentreich, 1976; Wender and Parker, 1976; Waith and Hirschhorn, 1978).

The properties just described led to increased interest and in the isolation of lectins from many sources (bacterial, fungi, snails and mammals). This appears to warrant an expansion of the term 'lectin' as it was frequently used to describe substances which differ markedly from those classically considered lectins, that is plant seed carbohydrate-binding proteins. As a result Goldstein et al., (1980) proposed a more strict definition of lectins according to which they should have the following properties:

(i) A lectin is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates.

(ii) Lectins should bear at least two sugar binding sites.

(iii) The specificity of a lectin should be defined in terms of monosaccharides (or simple oligosaccharides) that inhibit lectin-induced agglutination (or precipitation) reactions.

There are several other types of sugar-binding proteins including sugar-specific enzymes (glycosidases, glycosyltransferases...), transport protein, hormones (thyroid-stimulating hormone, follicle-stimulating hormone,...) and toxins (abrin, modeccin,...) etc. Under some conditions, sugar-specific enzymes with multiple combining sites can agglutinate cells (and/or precipitate glycoconjugates) and so act as lectins. On the other hand, in spite of similarities to true lectins from the same sources, toxins (which bear only one sugar combining site) should not be called lectins since they do not

agglutinate cells or precipitate glycoconjugates (Goldstein et al., 1980). More recently, Kocourek and Horejsi (1981) proposed the following definition of lectins: lectins are sugar-binding proteins or glycoproteins of non-immune origin which are devoid of enzymic activity towards sugars to which they bind and do not require free glycosidic hydroxyl groups on these sugars for their binding.

Lectins, their isolation, biological activities and applications have been reviewed by many authors (Lis and Sharon, 1973, 1977; Kornfeld et al., 1974; Sharon and Lis, 1975; Goldstein and Hayes, 1978; Lotan and Nicolson, 1979; Weir, 1980).

One of the most extensively investigated lectins, and the only one for which the primary sequence as well as the three-dimensional structure have been established is the lectin from jack bean(Canavalia ensiformis) concanavalin A (Con A). It was first isolated and crystallized by Sumner in 1919 and later its ability to precipitate polysaccharides have been studied in some detail by Sumner and Howell (1936), who were the first to suggest that haemagglutination by Con A may be the consequence of the reaction of the lectin with carbohydrates in stroma proteins. In addition to its ability to agglutinate cells and to precipitate glycoproteins and polysaccharides, Con A possesses strong mitogenic activity. Goldstein et al. (1964) established that the Con A binding site appears to require unmodified hydroxyl groups at positions C-3, C-4 and C-6 in the six-membered ring of α-mannopyranosides or α-glycopyranosides. Mannopyranosides and glycopyranosides bind to Con A in the C-1 chair conformation (Brewer et al., 1973) and it has been suggested (Poretz and Goldstein,

1970) that the protein has a specific site capable of interacting with the anomeric oxygen atom of the α-linked glycosides of these sugars. It is now known (Hardman and Ainsworth, 1972; Wang et al.,1971; Edmundson et al.,1971) that Con A consists of polypeptide subunits, of molecular weight 26,000. At pH 5.6 and below, two protomers are associated in a dimer of molecular weight 52,000; above pH 5.6, the dimer aggretate, forming tetramer. Con A exhibits also a reversible, temperature dependent dimer-tetramer transition(Gorden and Marquardt, 1974)

There is one binding site for saccharide per subunit (Yariv et al., 1968), as well as one for Mn^{2+} and one for Ca^{2+} (Kalb and Levitzki, 1968). Both metals are reversibly removed from the protein at low pH. The Mn^{2+} can be readily replaced by other transition metals, such as Ni^{2+} to yield fully active lectin preparations (Shoham et al., 1973). Occupation of the transition metal site is required for the binding of Ca^{2+}, and both appear to be necessary for the binding of saccharides (Kalb and Levitzki, 1968) although there is some uncertainty about the exact role of Ca^{2+}; according to one view, it is not absolutely necessary for saccharide binding but acts only by enhancing the rate of conformational change leading to the formation of the functional protein (Koenig et al., 1973; Richardson and Behnke, 1976). Stark and Sherry (1979) reported that a variety of individual metal ions or mixtures of ions added to demetallized Con A convert the protein to the conformational form which has a high affinity for saccharides with the rates of conversion dependent upon the identity of the ions added and the sample pH. They also concluded that Zn^{2+}, Co^{2+} and Pb^{2+} were able to fill both metal binding sites

and convert Con A to its active structure. Mn^{2+} and Ca^{2+} appear to stabilize the conformation of the protein,protecting it against heat inactivation (Doyle et al., 1976) and hydrolysis by proteolytic enzymes (Thomasson and Doyle, 1976).

Among the various applications of Con A is the isolation of Con A-binding glycoproteins from normal and transformed cells of various species (for review see Lotan and Nicolson, 1979)using Con A affinity columns, measurement of acute phase proteins in serum of man and animals during inflammation and tissue breakdown (Kohn et al., 1980), together with its wide use as a mitogen. Middlebrook et al. (1979) studied the interaction of Con A with diphtheria toxin and suggested that Con A could be used as a tool to study how the toxin crosses the cell membrane as Con A was found to prevent the internalization of the toxin into the cells. More details about the lectin can be found in a number of reviews (Cunningham, 1975; Bittiger and Schnebli, 1976; Goldstein and Hayes, 1978).

One of the lectins emphasised in this work is the peanut lectin (Arachis hypogaea), whose discovery was associated with the phenomenon of T-polyagglutinability which usually arises as a result of in vitro contamination of blood specimens through the degradive action of bacterial neuraminidase. This was first observed in 1927 by Thomsen who assumed that there exists a receptor L (latent), common to all red cells but normally Latent, which is activated or revealed through the effects of the bacterial 'agent' which was referred to as transforming bacterial enzyme by Friedenrich (1930). Thus the receptor was called T-receptor and the corresponding serum element

T-agglutinin. The bacterial enzyme was later identified as neuraminidase (Klenk, 1956; Gottschalk, 1960), but the nature of the T-receptor (T-antigen) on the red cells remained obscure, despite the fact that neuraminidase-treated red cell glycoproteins showed a strong inhibitory power towards the T-agglutinin (Klenk and Uhlenbruck, 1960). Following the work of other authors (Springer and Ansel, 1958; Mäkelä and Cantell,1958; Painter et al., 1962; Klenk et al., 1962) and from their own inhibition studies, Kim and Uhlenbruck (1966) were eventually able to determine the structure of the T-antigen to be the disaccharide β-D-galactopyranosyl (1→3)-N-acetyl-D-galactosamine. The T-antigen was later referred to as the Thomsen-Friedenrich antigen (Newman et al., 1977) to avoid any confusion with T for thymocyte or tumor antigen.

Bird (1964) was the first to extract from peanuts a T-agglutinin which resembled the naturally occurring antibody in having high affinity towards the disaccharide Gal-β-(1→3)-GalNAc. The peanut lectin was also found to be inhibited, to a lesser extent, by galactosides but not by the free N-acetylgalactosamine. The peanut lectin has been purified (Lotan et al., 1975; Terao et al., 1975) using affinity chromatography with a molecular weight of 110,000. The finding of a unqiue sequence for the five NH_2-terminal amino acids of the peanut lectin suggested four identical subunits (Lotan et al., 1975). The lectin was also shown to have no covalently bound carbohydrate. Dahr et al. (1974, 1975 c)showed that the TF-antigen is part of the alkali-labile tetrasaccharide that is involved in MN blood group activity. These findings confirmed the earlier report of Klenk and Uhlenbruck (1960) that the TF-antigen

is a cryptic antigen on human erythrocytic membrane glycoproteins and is usually covered by sialic acid. The lectin was found to be mitogenic (Novogrodsky et al., 1975) to human peripheral blood lymphocytes and to rat lymphocytes only after treatment of the cells with neuraminidase, but not to stimulate mouse and guinea pig lymphocytes. On the other hand, the lectin was reported (Terao et al., 1975) to have no mitogenic effects towards either native or neuraminidase treated human peripheral blood lymphocytes in spite of the similarity of the lectin preparations by both groups. The question of mitogenicity of the peanut lectin, therefore, remains unresolved.

The peanut lectin has been used for clinical determinations of T-polyagglutinability (Bird and Wingham, 1971) to detect surface TF-antigen on normal mammary epithelial cells (Klein, et al., 1978), to detect TF-antigen on blood group M and N substances (Dahr et al., 1975c)and glycoproteins isolated from bovine MFGM (Newman et al., 1976a). More recently, Reisner et al.(1980) reported that preleukaemic bone marrow and spleen cells of irradiated C57BL/6 mice that were inoculated with the radiation-induced leukaemia virus variant D-Rad LV differ from autonomous end-stage leukaemia cells in the expression of the receptor for peanut lectin. As a result, the preleukaemic cells are agglutinated by peanut lectin and the end-stage cells are not. They also demonstrated that the cells susceptible to D-Rad LV transformation in the thymus were present mainly among the peanut lectin-receptor-bearing cells.

Interest in the MN and precursor antigens has recently grown with the reports by Springer et al., (1975, 1977) that the TF-

antigen is demonstrable in human malignant breast tissue and that anti-TF antibody, which is present in all normal human sera, is severely depressed in a significant number of patients with cancer of the breast. Depression of anti-TF antibody was reported to be caused either by binding of antibody to TF-antigen on the surfaces of intact tumour cells or by the presence of antigen shed from the tumour cell membrane (Springer et al., 1977; Anglin et al., 1977). These findings were later contested by Newman et al. (1979) who showed no significant differerence in the levels of anti-TF antibody and TF-antigen in serum and tissue cultures of normal individuals and patients with breast carcinoma.

As has been discussed in the general introduction to this thesis, the milk fat globule membrane can be regarded as a model mammalian membrane and in particular a model for epithelial cell membranes of the donor species. In view of the known difference in recognition type interaction between corresponding membranes from different species (Newman et al., 1976a,b) it was of interest to compare the exposure of a range of individual monosaccharide residues on the surfaces of bovine and human milk fat globules. This approach is of special importance since most of the analytical studies on MFGM carbohydrate of various species have been concentrated on the extraction of glycoproteins by various methods from MFGMs (Newman et al., 1976a; Keenan et al., 1977; Newman and Uhlenbruck, 1977; Murray et al., 1979). Sugar residues exposed on the surface of intact milk fat globules may well be subjected to differential extraction or to conformational changes during purification of glycoprotein fractions while glycolipid components, are largely lost. It is clear that contribution of

carbohydrate structures to specific interactions of the MFGM and, by implication, also the mammary epithelial cell membrane, can most directly be studied in the intact fat globule.

This section describes a comparative study of the accessibility of various sugar residues on the surfaces of native and neuraminidase treated human and bovine milk fat globules. The study makes use of the fluorescence-activated cell sorter and of lectin-induced agglutination of milk fat globules.

Materials and Methods

Materials:

Arachis hypogaea (peanut agglutinin), *Ricinus communis* (type I and II), *Lens culinaris* (lentil lectin) concanavalin A (Con A), wheat germ agglutin, and *Lotus tetragonolobus*, both purified lectins and conjugated to fluorescein were all obtained from Sigma Chemical Co., London SW6, U.K. Native and fluorescein-conjugated phytohaemagglutinin (PHA) were generous gifts from Dr. R.A.Newman, Department of Immunology, Imperial Cancer Research Fund, London. Source plant material for soya bean, *Ulex europeus* and *Dolichos biflorus* lectins, extracted agglutinin from *Helix pomatia* and typed human red blood cells were generous gifts from Dr. D.J. Anstee, South West Regional Blood Transfusion Centre, Southmead, Bristol. *Vicia graminea* seeds were kindly supplied by Dr. W. Dahr, Department of Immunobiology, Medical University Clinic, Cologne, W. Germany. Phosphate buffered s.aline (PBS) tablets were obtained from Oxoid Ltd., Basingstoke, Hants, U.K. Microtiter plates were obtained from Sterilin Ltd., Teddington, Middlesex, U.K.

Neuraminidase [*Vibria comma* (cholerae)] was purchased from Behringwekre, Marburg/Lahn, W. Germany. All other reagents were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Fresh bovine milk was obtained from Friesian cows in mid-lactation and each sample was a pool from four individual animals. Human milk and blood were from individual volunteers in established lactation.

Methods:

Washed milk fat globules

Fresh milk (10 ml) was centrifuged at 15000 x g for 10 min at 22°C. The separated milk fat globules (cream) were resuspended in phosphate-buffered saline (PBS), pH 7.3 (10 ml) and recentrifuged under the same conditions. The washing procedure was repeated a further three times after which the milk fat globules were resuspended in PBS, pH 7.3, so as to give an optical density of approximately 1.7 O.D. when read against water at 700 nm.

Neuraminidase treatment of milk fat globules:

Washed milk fat globules (from 5 ml original milk) were suspended in PBS (1 ml), pH 5.6, containing 1 mM $CaCl_2$ and neuraminidase (50 µl, 1 IU/ml) and the mixture was incubated at 37°C for 30 min. The treated globules were removed by centrifugation at 15000 x g for 10 min at 22°C and resuspended in PBS, pH 7.3, (5 ml). The washing procedure was repeated a further three times after which the sialic acid-free globules were resuspended in PBS, pH 7.3, as described above.

Lectin-induced agglutination of milk fat globules:

A suspension of washed milk fat globules (20 µl) prepared as described above was mixed with lectin solution (20 µl) and PBS, pH 7.3, in a recessed microscope slide well and the agglutination was allowed to proceed at 22°C for 1 h. in a humid environment. The fat globules were examined at 100x magnification using a light microscope. Agglutination titres were expressed as the reciprocal of the minimum dilution of lectin giving agglutination of less than 30%. Control experiments were carried out by using buffer in place of lectins.

Inhibition studies were performed by incubating the lectin solution (20 µl) (diluted 2^{x-3}, where 2^x is the agglutination titre) with inhibitor (monosaccharides or MFGM-derived glycopeptides) containing buffer (20 µl) for 30 min at 22°C prior to addition of milk fat globule suspension (20 µl). The mixture was allowed to stand for 1 h at 22°C in a humid environment after which the fat globules were examined at 100x magnification using a light microscope. Agglutination titres are expressed as the reciprocal of the minimum dilution of lectin giving agglutination of less than 30% of the total fat globules.

Analysis of human and bovine milk fat globules by the fluorescence Activated cell sorter

I. The instrument:

The fluorescence activated cell sorter (FACS) is an automatic electronic device of potentially great importance. It is capable of identifying and separating functionally distinct groups of viable cells that have, or can be made to have, either different fluorescence intensities, different light-scattering characteristics, or different combinations of these two variables. The instrument, its various modes of operation and its application have been described by many authors (Bonner et al., 1972; Hulett et al., 1973; Greaves et al., 1976).

A simplified diagram illustrating the general principles is shown in Fig. 49. The cells are suspended in saline and emerge essentially in single file from an ultrasonically vibrated nozzle. The cell suspension breaks up into regularly-spaced droplets forming

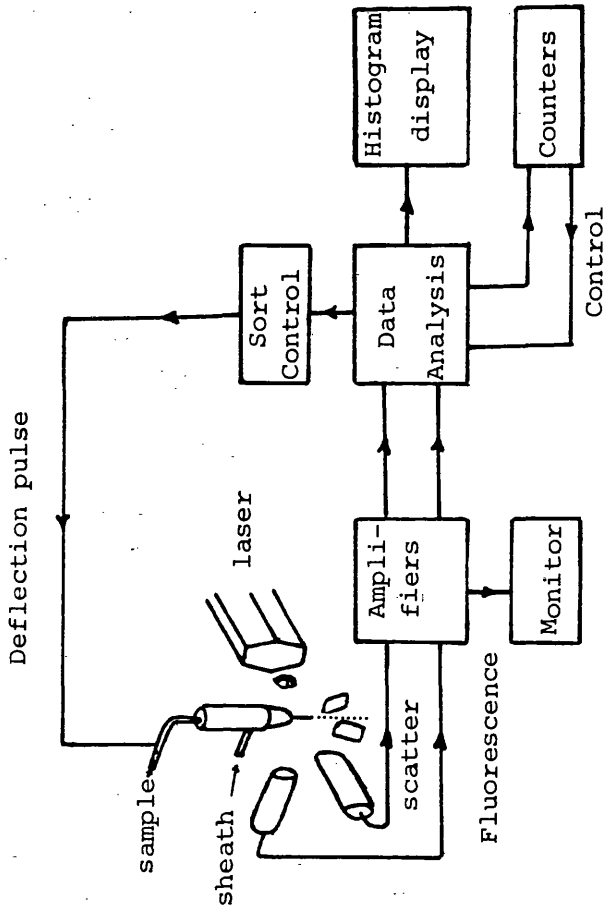


Fig. 49. Schematic representation of the FACS

a stream of 50 μ diameter which is intersected by a beam from an argon-ion laser. As individual cells pass through the beam they scatter some of the light and, if labelled, they also fluoresce. The two types of signals from the cells are detected separately (using microscope objectives), amplified and converted into voltage pulses the size of which is proportional to the input signal. After the pulse hiehgt analysis, these data are displayed in the form of a histogram (see later); the ordinates representing the number of events (i.e. cells) recorded against pulse height (i.e. cell fluorescence intensity or size). Cells having particular characteristics of interest can be both enumerated and physically separated from the remainder. Separation is achieved by imparting a charge to the droplet stream. By 'informing' the FACS of the characteristics of the cells to be separated and by the use of finely controlled timing, droplets containing cells of interest can be charged and deflected into collection tubes, as they fall between charged plates (Fig. 49). Data are presented as polaroid photographs of the storage display following analysis of 10,000 globules. Standard settings of the FACS were, Laser: 200 mW, Photomultiplier tube; 760 v, fluorescence gain: 16/0.1 and scatter gain; 2/1.

II. Sample preparation

Native or neuraminidase-treated milk fat globule suspension (100 μl) prepared as described above, was incubated at 22°C with fluorescein-labelled lectin solution (50 μl) and PBS buffer, pH 7.3, (20 μl) for 30 min, after which more PBS buffer, pH 7.3, (2 ml) was added. The globules were then separated by centrifugation at 15000 x g for 10 min and resuspended in PBS buffer, pH 7.3, (1 ml) before examination by the FACS.

Inhibition studies were performed by incubating fluorescence-labelled lectin solution (50 µl) with inhibitor-containing buffer (20 µl) for 30 min at 22°C after which globule suspension (100 µl) was added and the mixture was allowed to stand at 22°C for 30 min before separating and resuspending the globules in buffer (1 ml) for examination by the FACS as described above. Inhibitor concentrations were 0.1 M in the case of monosaccharides and 1 mg/ml in the case of inhibition by MFGM-derived glycopeptides.

Lectin Solutions:

Commercially-obtained lectins were dissolved in PBS buffer, pH 7.3 , to an initial concentration of 2 mg/ml except in the case of Lotus tetragonolobus when 1 mg/ml were used. Samples were serially diluted (2x) with PBS buffer, pH 7.3, for agglutination assays. For FACS analysis aliquots (50 µl) of fluorescein-labelled lectin solution containing lectin (150 µg) were used.

For lectins extracted directly from plant material, dry source material (1 g) was ground in a mortar and added to PBS buffer, pH 7.3 (10 ml) at 4°C. The suspension was homogenized (MSE bench homogenizer, 6 x 30 sec) at 4°C and allowed to stand at 4°C for a further 5 min before centrifugation at 14,000 x g for 10 min at 4°C. The aqueous supernatant was extracted (3 x 1 vol.) with diethyl ether, separated and freed from residual ether in a stream of N_2 at 4°C.

The activities of all lectin solutions were checked by demonstrating haemagglutination of neuraminidase-treated type O,NN red blood cells

(or native type A cells in the case of Helix pomatia and Dolichos biflorus) by high dilutions of the lectin solution (Table 17). Specificities were confirmed by showing complete inhibition of haemagglutination caused by four haemagglutination doses of lectin when a 10 mM-solution of the immunodominant sugar (20 µl) was incubated with the lectin solution for 30 min at 22°C prior to addition of red blood cells in the haemagglutination assay. The specificity of agglutination of milk fat globules was also checked directly by similar inhibition of three agglutination doses of lectin by 30 mM solutions of the respective immunodominant sugar. In the case of Arachis hypogaea specificity of milk fat globule agglutination was demonstrated by inhibtion of three agglutination doses of the lectin by 10 mM concentrations of purified Thomsen-Friedenreich antigen.

Haemagglutination assays:

Samples of human red blood cells (50% suspension in acid-citrate dextrose anti-coagulant) were diluted (to 10 ml) in PBS buffer, pH 7.3, and centrifuged at 3,500 x g for 10 min. The pelleted cells were resuspended in PBS and again centrifuged. The washing procedure was repeated three times after which, pelleted cells were diluted (to 3% v/v) in PBS buffer, pH 7.3, before being used in agglutination studies.

Neuraminidase-treated cells were prepared by suspending pelleted native red blood cells (0.5 ml) in PBS buffer, pH 5.6, (4.5 ml) containing 1 mM $CaCl_2$ and neuraminidase (50 µl, 1 IU/ml) and the mixture was incubated at 37°C for 30 min. Cells were freed from neuraminidase and free sialic acid by repeated (3x) centrifugation

Lectin	Typed human red blood cells	
	O, NN	A, MN
Ricinus communis (type I) (galactose, N-acetylgalactosamine)	2^{15}	2^{15}
Lens culinaris (mannose, glucose)	2^{11}	2^{11}
Lotus tetragonolobus (fucose)	2^{5}	2^{5}
Helix pomatia (N-acetylgalactosamine)	2^{2}	2^{12}
Dolichos biflorus (N-acetylgalactosamine)	neg	2^{6}
Ulex europus (fucose)	2^{3}	2^{3}
Vicia graminea (in part TF antigen)	2^{9}	2^{3}
Arachis hypogaea (TF antigen)	neg	neg

Table 17. Shows the activities of some of the lectins used with erythrocyte indicator cells. Titres are expressed as the reciprocal of the minimum dilution of lectin resulting in no agglutination of 3% suspension of cells.

at 3,500 x g for 10 min and resuspended in PBS (pH 7.3, 10 ml). Final packed washed cells were suspended (3% v/v) as described above for agglutination studies.

For the haemagglutination assay, tested cells, prepared as described above (20 µl suspension) were mixed with PBS buffer pH 7,3 (20 µl) and serially diluted lectin solution (20 µl) and allowed to stand in the well of a microtitre plate for 1 h at 26°C. Absence of agglutination was indicated by the presence of a compact 'button' of cells at the bottom of the well.

Isolation of sialoglycopeptides from bovine MFGM:

Sialoglycopeptides were cleaved off intact washed bovine milk fat globules by treatment with pronase as described in Section A. The pronase digested membrane materials were fractionated on Sephadex G-50 to give a major excluded peak which when fractionated by ion-exchange chromatography gave two fractions SP and SR (Section A, p. 72). The SR glycopeptide fraction was shown to contain high concentrations of the unsubstituted TF-antigen. Treatment of this fraction with mild acid (0.2 N H_2SO_4 at 80°C for 1 h) followed by chromatography on Sephadex G-25 resulted in the isolation of a purified sialic acid-free fraction containing approximately 0.3 mM of the free disaccharide β-D-galactopyranosyl-(1→3)N-acetyl-D-galactosamine (TF-antigen) (Section A, p. 83).

Preparation of free Thomsen-Friedenrich antigen:

As shown in Section A, treatment of the sialic acid rich (SR) glycopeptide (Fig. 27a) with alkaline borohydride resulted in the release of the alkali labile tetrasaccharide N-acetylneuraminyl-(2→3)-

β-D-galactopyranosyl-(1→3) [N-acetylneuraminyl-(2→6)] -N-acetyl-D-galactosaminitol (Peak III, Fig. 34). Fractions corresponding to the tetrasaccharide peak were combined and freeze dried to give a residue (0.5 - 1 mg). Combined residual tetrasaccharide material (5 mg) were incubated with 0.2 N H_2SO_4 (1.5 ml) at 80° C for 1 h. The hydrolysate was applied to the top of previously equilibrated column of DEAE-Sephadex A-25 (16 x 1.3 cm). The column was eluted with 50 mM pyridine acetate buffer, pH 5, (100 ml) followed by a linear gradient (50 - 650 mM) of pyridine acetate (200 ml) at 22°C with a flow rate of 40 ml/h. Fractions (5 ml) were automatically collected and aliquots (0. 1 ml) were assayed for sialic acid and hexose as described in Section A. The elution profile is shown in Fig. 50. Fractions corresponding to peak I (Fig. 50) were collected and freeze-dried to give the disaccharide β-D-galactopyranosyl-β-D-(1→3) N-acetyl-D-galactosaminitol (≈2 mg).

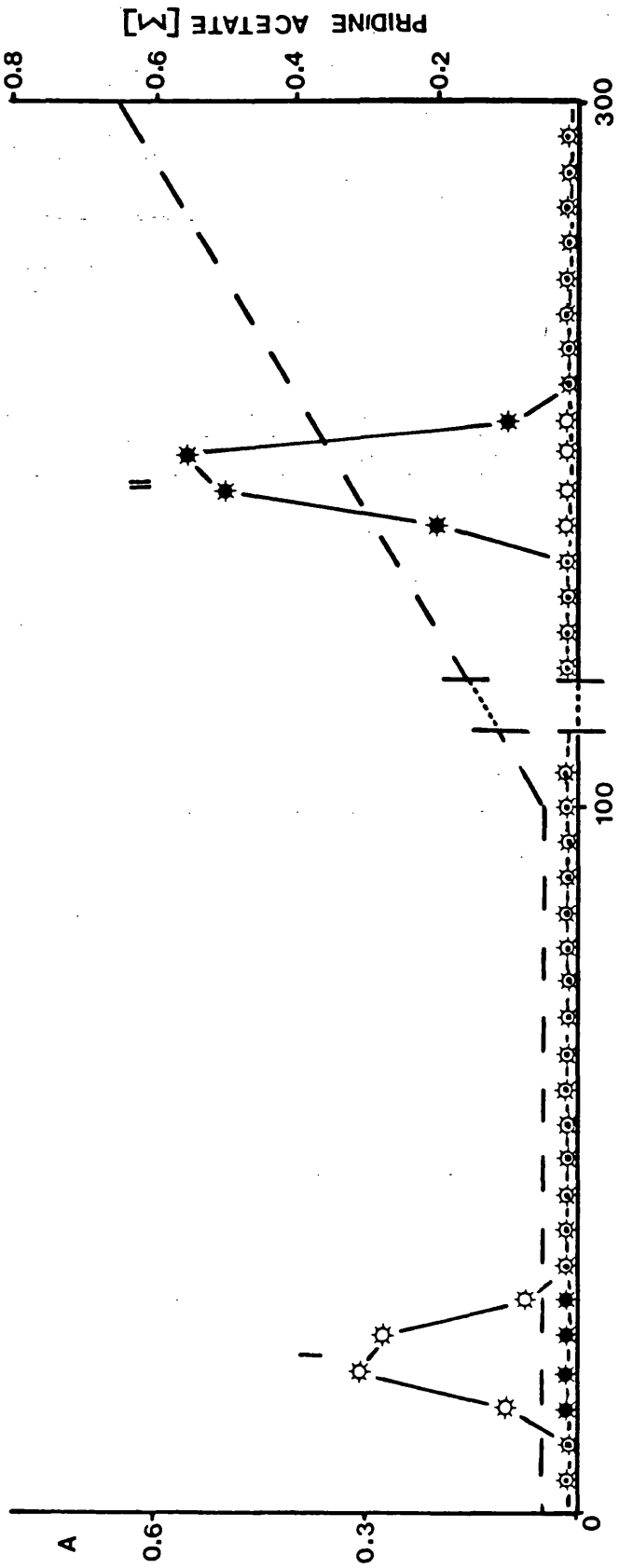


Fig. 50. Separation of the free (reduced)TF antigen from free sialic acid on a column of DEAE-Sephadex A-25. The column was eluted with 0.05M-pyridine acetate buffer (100 ml) pH 5.0 followed by a linear gradient of 0.05 - 0.65 M-pyridine acetate buffer (200) pH 5.0. Aliquots (0.1 ml) of fractions (5 ml) were individually assayed for hexose (A_{420} ☼) and sialic acid (A_{550} ✸).

Results

I. Lectin-induced agglutination of human milk fat globules

Native and neuraminidase treated human milk fat globules were subjected to agglutination by a range of purified lectins using the assay described in the Methods section and the average titres obtained are shown in Table 18. Titres represent the means of 20 samples from different individuals of varying ABO and MN blood groups.

The individual titres obtained from a number of donors by using the peanut agglutinin (PNA), Arachis hypogaea, are shown in Table 19. The data shown in Table 19 clearly demonstrates that unlike human blood cells, which are agglutinated by PNA only after treatment with neuraminidase, human milk fat globules from healthy individuals are readily agglutinated by high dilutions of PNA irrespective of the blood group of the donor with no significant increase of the titre after neuraminidase treatment of the globules. These findings indicate that in contrast to normal human red blood cells, receptors of PNA at the surfaces of normal human milk fat globules are almost totally unsubstituted with sialic acid. Thus, high concentrations of the unsubstituted Thomsen-Friedenrich (TF) antigen (β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosamine) are exposed at the surfaces of normal human milk fat globules, and accessible to the lectin. Indeed, this was confirmed by inhibition of PNA-induced agglutination of human milk fat globules by 10 mM concentrations of the purified (Materials and Methods) TF-antigen and by glycopeptide fraction (Section A, Fig. 31) containing 0.3 mM of the unsubstituted (free) TF-antigen.

Lectin	HUMAN MILK FAT GLOBULES NATIVE WASHED	NEURAMINIDASE-TREATED
Ricinus communis type I (galactose, N-acetylgalactosamine)	2^{10}	2^{10}
Ricinus communis type II (galactose)	2^{8}	2^{8}
Soya bean agglutinin (galactose)	2^{5}	2^{7}
Concanavalin A (mannose/glucose)	2^{8}	2^{10}
Lens culinaris (mannose/glucose)	2^{8}	2^{9-10}
Wheat germ agglutinin (N-acetylglucosamine)	2^{8}	2^{8-9}
Ulex europaeus (fucose)	neg	neg
Lotus tetragonolobus (fucose)	neg	2^{o}
Arachis hypogaea (Thomsen-Friedenreich antigen)	2^{7-8}	2^{7-8}
Vicia graminea (in part Thomsen-Friedenreich antigen)	neg	neg
Helix pomatia (N-acetylgalactosamine)	neg	2^{3}
Dolichos biflorus (N-acetylgalactosamine)	neg	neg

* Values are the means of titres obtained by using 20 samples of milk all from different individuals of varying ABO and MN blood groups. No differences in titre were observed with different blood groups

Table 18. Agglutination titres of human milk fat globules with different lectins

	Normal Human Milk																Human Blood Cells		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	1	2	3
ABO type	A	O	A	O				A	O	A	A		O		AB	O	O	O	O
MN type	MN	MN	MN	MN				MN	MN	MN	MN		MN		MN	MN	MN	MN	N N
Washed globules/ cells	2^6	2^6	2^6	2^6	2^7	2^7	2^6	2^6	2^7	2^6	2^7	2^6	2^7	2^6	2^7	2^6	-	-	-
Neuraminidase treated globules/ cells	2^7	2^6	2^6	2^7	2^7	2^7	2^7	2^6	2^6	2^6	2^7	2^6	2^7	2^6	2^7	2^6	2^{11}	2^{12}	2^{12}

Table 19. Titres of peanut lectin-induced agglutination of human milk fat globules, and for comparison human red blood cells. Titres are expressed as the reciprocal of the minimum dilution of lectin giving agglutination of less than 30% of the total fat globules.

As shown in Table 18, human milk fat globules were not agglutinated by the fucose-specific lectins, Ulex europeus and Lotus tetragonolobus although weak agglutination was detected with the latter lectin following neuraminidase treatment of the human globules. Human milk fat globules were not agglutinated either by Helix pomatia or by Dolichos biflorus lectins irrespective of the blood group of the donor. Helix pomatia, however, was able to agglutinate human globules following their treatment with neuraminidase (Table 18). Vicia graminea failed to agglutinate human milk fat globules even after their treatment with neuraminidase (Table 18), which indirectly supports the results obtained with PNA (Vicia graminea requires the TF-antigen in part in its receptor). Table 18 also shows that human milk fat globules were specifically agglutinated by high dilutions of Ricinus communis (type I and II) soya bean, concanavalin A, Lens culinaris and what germ agglutinin, demonstrating the accessibility of galactose (Ricinus type I and II, soya bean), mannose and/or glucose (Con A, Lens lectins) and N-acetylglucosamine (wheat germ agglutinin) on the surfaces of intact human milk fat globules. Of these lectins, soya bean, Con A, Lens and to a lesser extent WGA showed increased activity following treatment of the globules with neuraminidase (Table 18).

II. Lectin-induced agglutination of bovine milk fat globules:

Native and neuraminidase treated bovine milk fat globules were agglutinated by a range of purified lectins as described in the Methods Section(p. 152) and the average titres are shown in Table 20. Titres represent means of experiments using five batches of pooled milk each of which was pooled from four individual cows.

Lectin	BOVINE MILK FAT GLOBULES*	
	Native, washed	Neuraminidase-treated
Ricinus communis type I (galactose, N-acetylgalactosamine)	2^6	2^8
Ricinus communis type II (galactose)	2^7	2^9
Soya bean agglutinin (galactose)	2^7	2^9
Concanavalin A (mannose/glucose)	2^{4-5}	2^{7-8}
Lens culinaris (mannose/glucose)	2^1	2^{2-3}
Wheat germ agglutinin (N-acetylglucosamine)	2^8	2^8
Ulex europaeus (fucose)	neg	neg
Lotus tetragonolobus (fucose)	neg	neg
Arachis hypogaea (Thomsen-Friedenreich antigen)	2^{2-3}	2^{5-6}
Vicia graminea (in part Thomsen-Friedenreich antigen)	neg	2^{6-7}
Helix pomatia (N-acetylgalactosamine)	2^9	2^{10}
Dolichos biflorus (N-acetylgalactosamine)	2^4	2^6

*Values are the means of titres obtained by using 5 samples of milk each of which was pooled from 4 individual cows

Table 20. Agglutination titres of bovine milk fat globules with different lectins.

Pooled bovine milk fat globules were found to be agglutinated by high dilutions of Ricinus communis (types I and II), soya bean and what germ agglutinins, Concanavalin A and Helix pomatia lectin. In all cases agglutination was specifically inhibited by the corresponding monosaccharide receptor (Table 20). Specific agglutination by these lectins demonstrates the accessibility of galactose (Ricinus types I and II, soya bean), mannose and/or glucose (Concanavalin A), N-acetyl-D-glucosamine (wheat germ agglutinin) and N-acetyl-D-galactosamine (Helix pomatia) on the surfaces of intact bovine milk fat globules. As with human globules, bovine milk fat globules were not agglutinated by the fucose-specific lectins, Ulex europeus and Lotus tetragonolobus, although fucose was detected in bovine MFGM-derived glycopeptides (Section A, p. 88). Bovine milk fat globules were agglutinated by Lens and PNA to a lesser extent than human globules and in both cases the agglutination titres were increased following treatment of the globules with neuraminidase (Table 20). Thus the TF-antigen at the surfaces of bovine milk fat globules is apparently present both free and substituted with sialic acid. These findings agree with the agglutination titres obtained with Vicia graminea which agglutinated bovine globules only after treatment of the latter with neuraminidase (Table 20).

III. Fluorescence activated cell sorter analysis of the interaction of lectins with human MFGs:

The degree of interaction of each of a range of purified lectins with native and neuraminidase-treated normal human milk fat globules was assessed by examining the percentage of globules carrying fluorescent-labelled lectins as described in the Methods section.

The results obtained are shown in Table 21 which demonstrates a high percentage of fluorescent-labelling of the human globules by fluorescein-labelled PNA, Ricinus (type II), soya bean agglutinin and wheat germ agglutinin. The percentage of labelling increased after neuraminidase treatment of the globules in the case of Ricinus and soya bean lectins but no significant increase was observed in the case of PNA (Fig. 51 A and B) and wheat germ agglutinins (Table 21). No interaction between the human globules and phytohaemagglutinin was observed even after treatment of the globules with neuraminidase (Fig. 52 A and B). These findings agree with those obtained from the milk fat globule agglutination assay (Table 18), and, indeed, stresses the sensitivity of the agglutination assay.

The labelling of the globules with lectins listed in Table 21 was specifically inhibited by 30 mM concentrations of the respective receptor of each lectin.

Fluorescence activated cell sorter analysis of the interacation of lectins with bovine MFGs:

The degree of interaction of each of a range of purified lectins with native and neuraminidase treated bovine milk fat globules was assessed by examining the percentage of globules labelled with fluorescent lectins as described for human globules. The results obtained (Table 22) were in general consistent with those obtained from the milk fat globule agglutination assay (Table 20). As shown in Table 22, pooled bovine milk fat globules were highly labelled by fluorescent Ricinus (type II), wheat germ agglutinin. Unlike human globules, bovine milk fat globules were poorly labelled with

Lectin	NORMAL HUMAN MILK FAT GLOBULES	
	Native, washed	Neuraminidase-treated
Ricinus communis type II (galactose)	90% ± 1	97% ± 1
Soya bean agglutinin (galactose)	80% ± 1	88% ± 1
Arachis hypogaea (TF-antigen)	88% ± 1	90% ± 1
Wheat germ agglutinin (N-acetyl-D-glucosamine)	76% ± 1	78% ± 1
Phytohaemagglutinin (N-acetyl-D-galactosamine)	-ve	-ve

Table 21. Percentage of fluorescence-labelling of native and neuraminidase treated normal human milk fat globules following their incubation with a range of fluorescent-labelled lectins.

Lectin	POOLED BOVINE MILK FAT GLOBULES	
	Native, washed	Neuraminidase-treated
Ricinus communis type II (galactose)	82% ± 1	89% ± 1
Soya bean agglutinin (galactose)	50% ± 1	72% ± 1
Arachis hypogaea (TF-antigen)	10% ± 1	40% ± 1
Wheat germ agglutinin (N-acetyl-D-glucosamine)	72% ± 1	73% ± 1
Phytohaemagglutinin (N-acetyl-D-galactosamine)	-ve	- ve

Table 22. Percentage of fluorescence-labelling of native and neuraminidase treated bovine milk fat globules following their incubation with a range of fluorescent-labelled lectins.

fluorescent PNA, but the percentage of labelling was dramatically increased (3 fold) following treatment of the globules with neuraminidase (Fig. 51C and D; Fig. 53) suggesting that the lectin receptor (TF-antigen) is largely substituted with sialic acid. Percentages of labelling of the bovine globules were also increased after their treatment with neuraminidase in the case of fluorescent soya bean agglutinin (Fig. 54) and to a lesser extent by fluorescent Ricinus (type II). As in the case of the human globules, bovine milk fat globules did not interact with phytohaemagglutinin even after their treatment with neuraminidase (Fig. 52 C and D). The interaction of bovine milk fat globules with the lectins listed in Table 22 were specifically inhibited by 30 mM concentrations of the respective receptor of each lectin.

Inhibition by bovine-derived sialoglycopeptides of the interaction of specific lectins with human milk fat globules:

The ability of each of a range of bovine MFGM-derived sialoglycopeptides (Section A) to inhibit the attachment of specific lectins to human milk fat globules was assessed by using the fluorescent activated cell sorter as described in the Methods section. The results are shown in Table 23. As can be seen from Table 23, the partially purified (PP) sialoglycopeptides mixtures (Section A) completely or partially inhibited the interaction of human milk fat globules with fluorescent soya bean agglutinin, PNA, WGA, Ricinus (type II) and Helix pomatia lectins. Mannose and/or glucose (Con A) and N-acetyl-D-glucosamine (WGA)-specific lectins were best inhibited by the SP-glycopeptide fraction indicating that the carbohydrate residues in this fraction are accessible to

Fig. 51. Fluorescence Activated Cell Sorter (FACS) analysis of peanut lectin binding to human and bovine milk fat globules.

Ordinate: relative fluorescence intensity.

Abscissa: relative globule size.

A. Lectin incubated with native human globules.

B. Lectin incubated with neuraminidase-treated human globules.

C. Lectin incubated with native bovine globules.

D. Lectin incubated with neuraminidase-treated bovine globules.

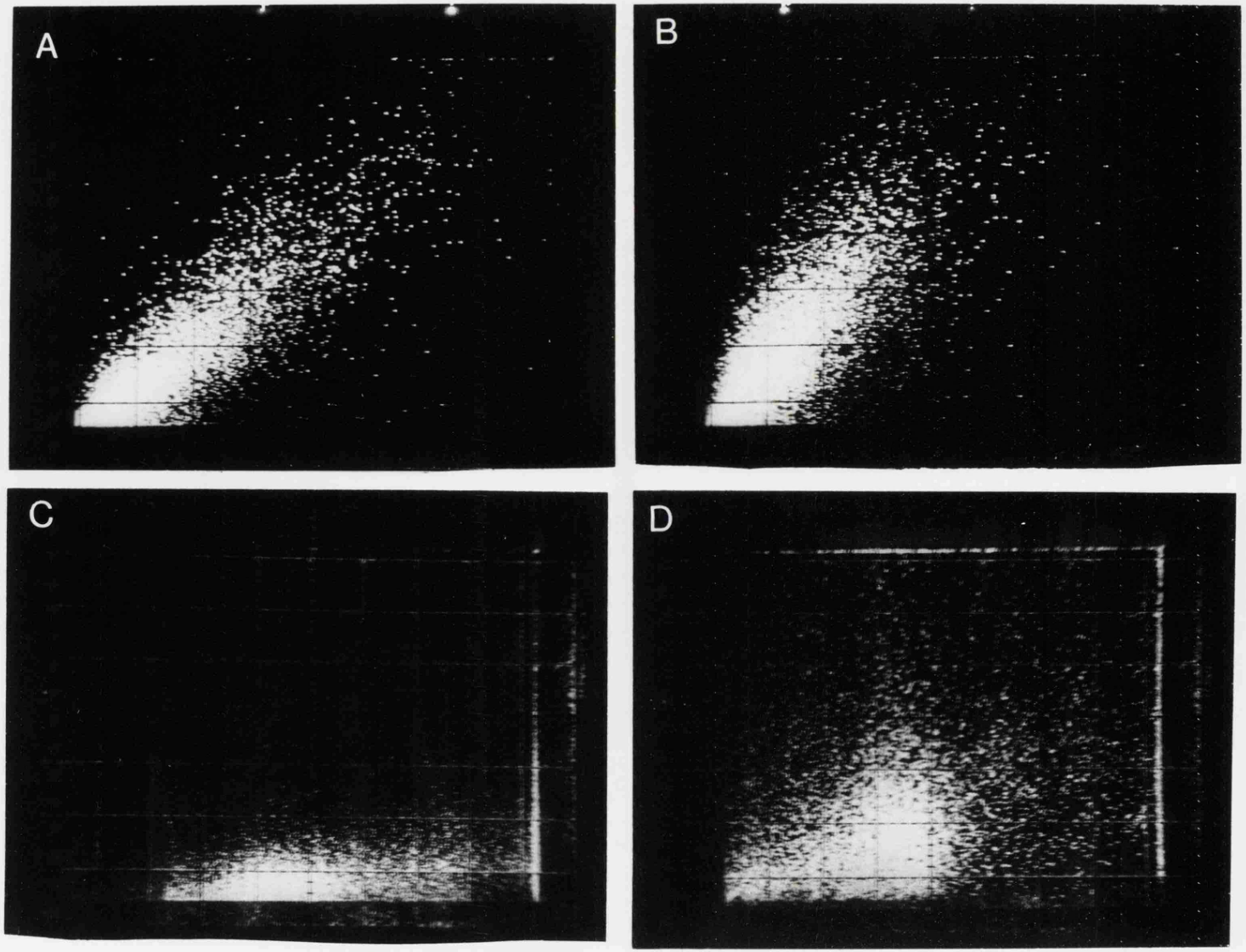

A
B
C
D

Fig. 52. FACS analysis of phytohaemagglutinin binding to human and bovine milk fat globules.

Ordinate: relative fluorescence intensity

Abscissa: relative globule size

A. Intact human milk fat globules.

B. Neuraminisae-treated human globules.

C. Intact bovine milk fat globules.

D. Neuraminidase-treated bovine globules.

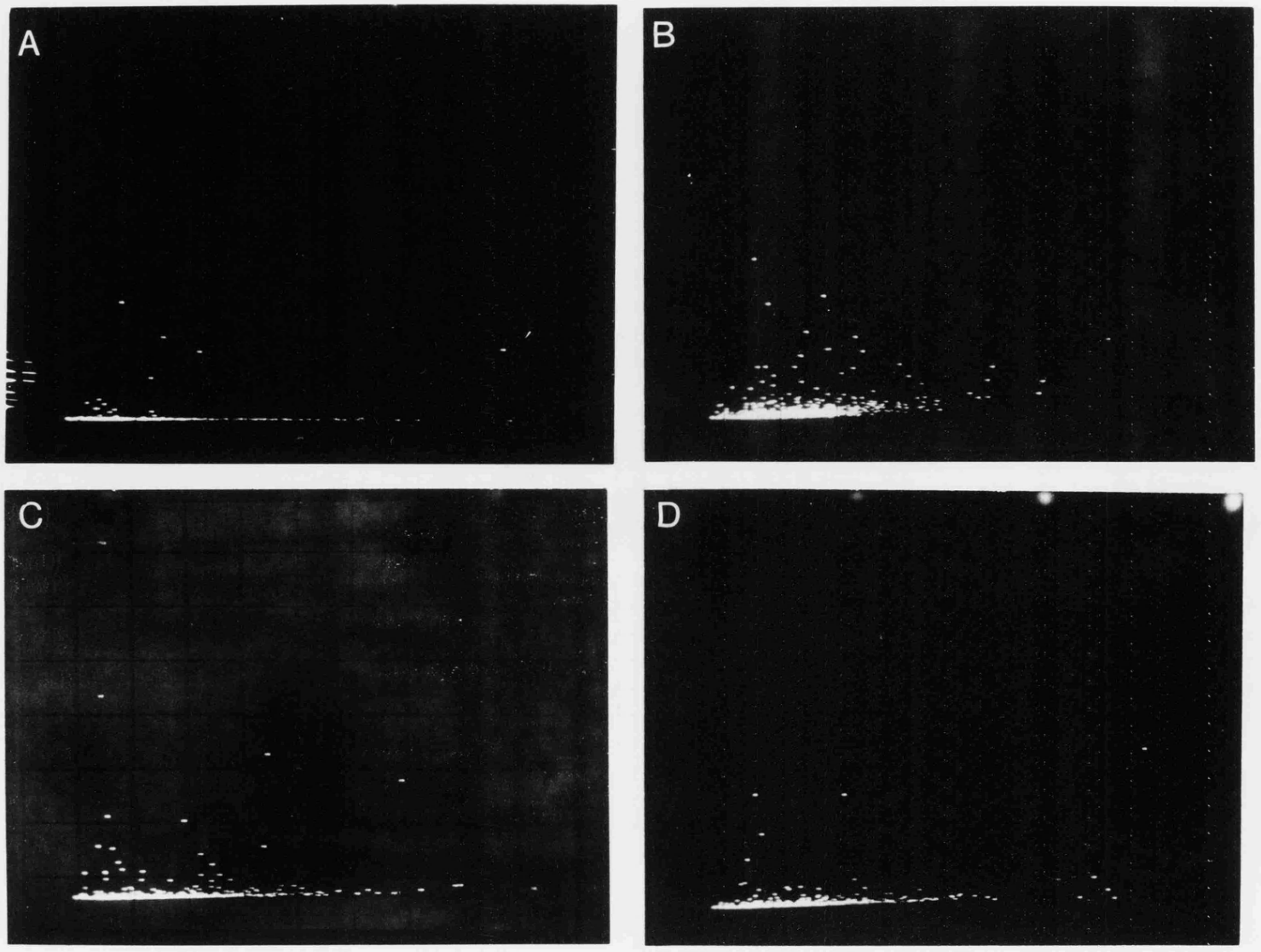

A
B
C
D

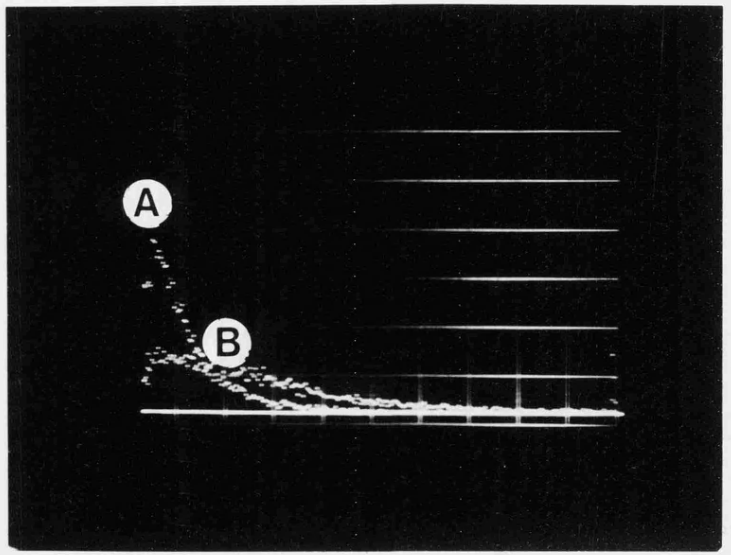
Fig. 53. FACS analysis of peanut lectin to bovine milk fat globules.

Ordinate: relative globule number

Abscissa: relative fluorescence intensity.

A. Lectin incubated with native globules

B. Lectin incubated with neuraminidase-treated globules.


A
B

these lectins (Fig. 56 and Table 23). On the other hand, the SR-glycopeptide fraction did not inhibit the interaction of fluorescent WGA and of fluorescent Ricinus II with human globules but inhibited the globules interaction with fluorescent Con A to a much lesser extent than the inhibition obtained using the SP-glycopeptide fraction. The SP-glycopeptide fraction also inhibited the interaction of the human globules with fluorescent soya bean agglutinin. In fact, soya bean interaction with the human globules was completely inhibited by all glycopeptides used (Table 23), demonstrating the accessibility of galactose residues in these glycopeptides to the lectin. Arachis (PNA) interaction with the human globules was only inhibited significantly by the acid hydrolysed SR glycopeptide fraction (Fig. 57) (sialic acid free) which was shown to contain approximately 0.3 mM of the free TF-antigen (Section A). The PP-glycopeptide mixture inhibited fluorescent PNA interaction with the human globules by only 20% (Table 23) which could account for the interaction of PNA with galactose as the lectin is known to have affinity towards galactose. To overcome the argument that PNA binds galactose rather than the TF-antigen, competitive binding experiments were carried out using fluorescent Ricinus (type II) together with non-labelled PNA and vice versa. The percentages of labelling of the human globules in this experiment (Table 24) shows that each of Ricinus (type II) and PNA attaches to different receptors at the surfaces of intact human milk fat globules. Indeed, when inhibition studies were carried out by using galactose and TF-antigen the results showed that 10 mM of the TF-antigen were required to completely inhibit PNA interaction with the human globules while 33 mM of galactose were required to demonstrate the same inhibition. This

Lectin \ Inhibitor	SR glycopeptide	SP glycopeptide	PP glycopeptide	Alkaline borohydride-treated SR glycopeptide	Desialylated SR glycopeptide
Soya bean agglutinin	100%	100%	100%	100%	100%
Arachis hypogaea	neg	neg	20%	neg	100%
Ricinus-II	neg	neg	70%	50%	50%
Con A	neg	30%	60%	65%	50%
Helix pomatia	neg	neg	neg	neg	neg
Phytohaemagglutinin	neg	neg	neg	neg	neg
Wheat germ agglutimin	neg	50%	50%	neg	neg

Table 23. Inhibition by bovine MFGM-derived sialoglycopeptides of fluorescent labelling of human milk fat globules by a range of lectins.

	A^+	A^+,R^-	R^+	R^+,A^-
% of labelled globules (1)	75%	72%	88%	80%

A^+ = fluorescent Arachis

A^- = non-labelled Arachis

R^+ = fluorescent Ricinus (type II)

R^- = non-labelled Ricinus (type II)

Table 24. Competitive binding of Ricinus and Arachis lectins to human milk fat globules.

strongly suggests that the binding of PNA to human milk fat globules is through the TF-antigen rather than galactose.

The SR-glycopeptide fraction failed to inhibit Ricinus (type II) which has high affinity towards galactose indicating that receptors of this lectin in the glycopeptide are probably masked by sialic acid. Indeed this was shown to be the case when 50% inhibition was achieved by using the sialic acid free SR-glycopeptide fraction and by the alkaline borohydride treated SR-glycopeptide fraction (Fig. 55).

Fig. 54. FACS analysis of soya bean agglutinin binding to bovine milk fat globules.

Ordinate: relative globule number.

Abscissa: relative fluorescence intensity.

A. Lectin pre-incubated with galactose.

B. Lectin incubated with native globules.

C. Lectin incubated with neuraminidase-treated globules.

Fig. 55. FACS analysis of Ricinus-II binding to human milk fat globules.

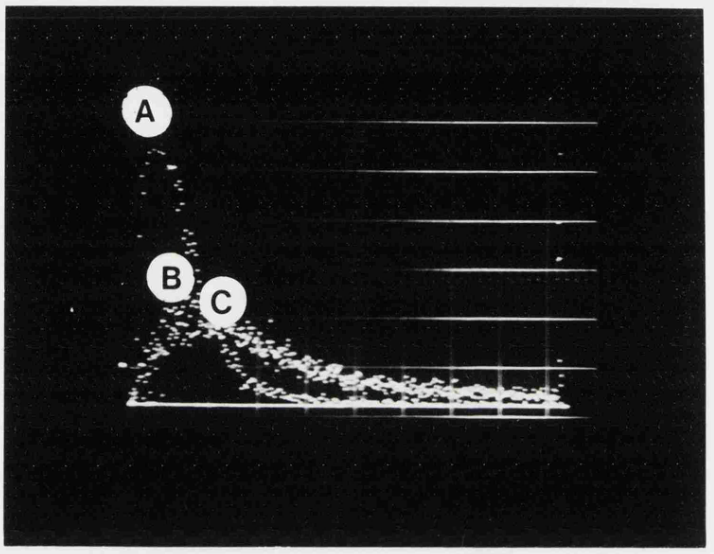
Ordinate: relative globule number.

Abscissa: relative fluorescence intensity.

A. Lectin pre-incubated with galactose.

B. Lectin pre-incubated with desialylated SR glycopeptide.

C. Lectin incubated with native globules.


A
B
C

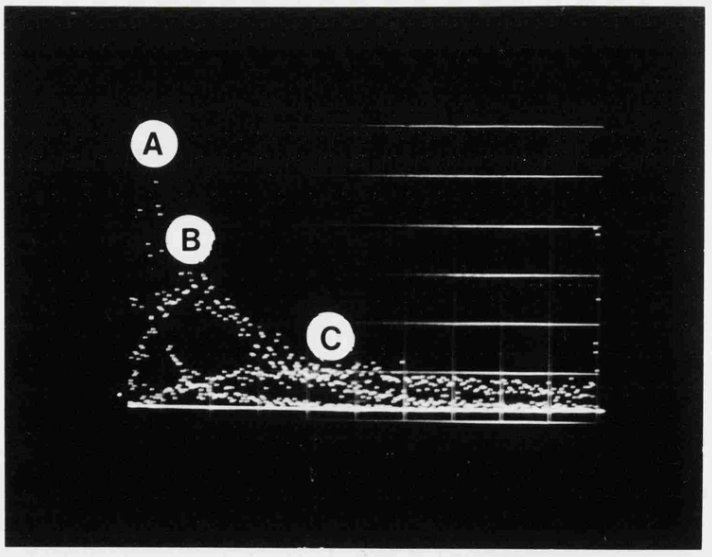

A
B
C

Fig. 56. FACS analysis of con A binding to human milk fat globules.

Ordinate: relative globule number.

Abscissa: relative fluorescence intensity.

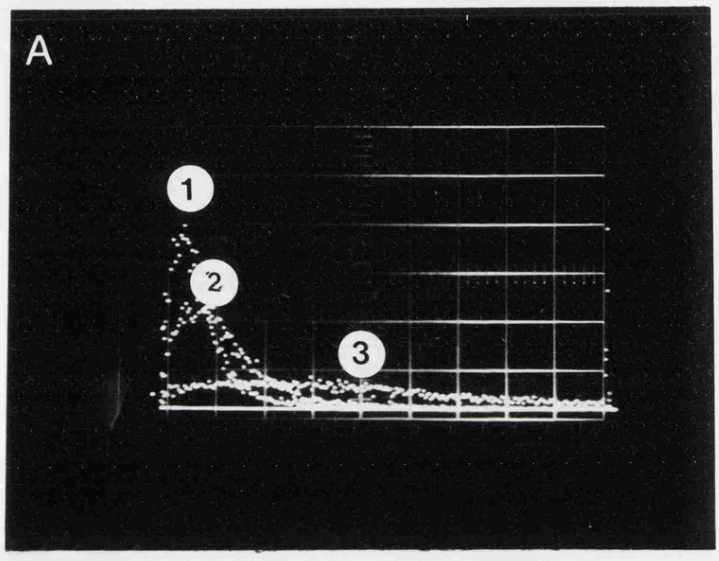
A. 1. Con A pre-incubated with SP glycopeptide

2. Con A pre-incubated with SR glycopeptide

3. Con A.

B. 1. Con A pre-incubated with mannose

2. Con A.


A
1
2
3

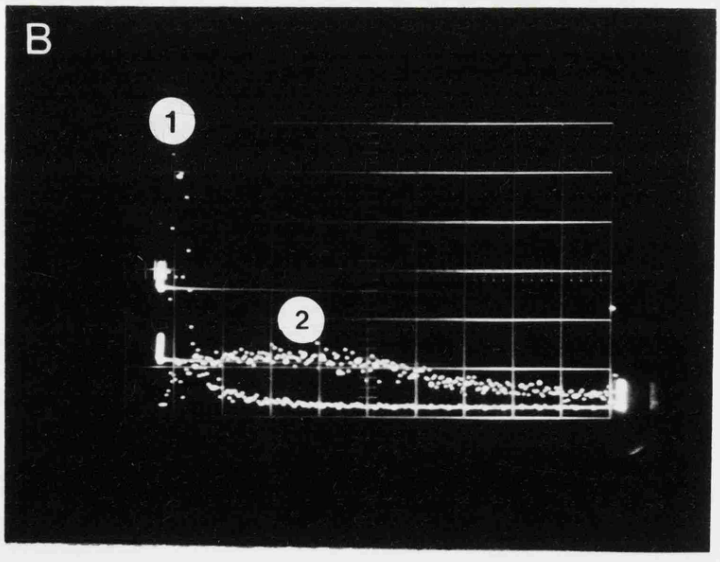

B
1
2

Fig. 57. FACS analysis of peanut lectin binding to human milk fat globules.

Ordinate: relative fluorescence intensity

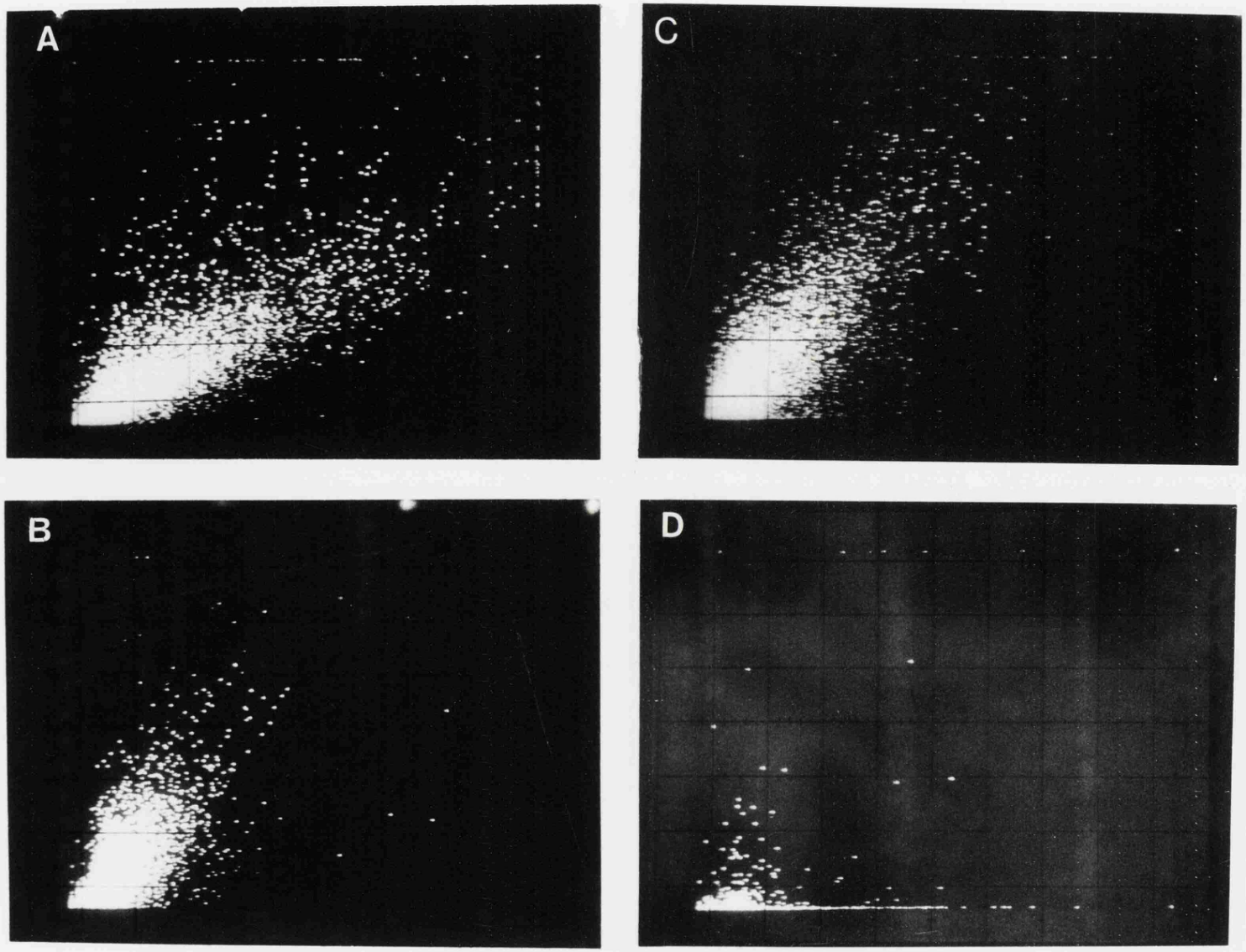
Abscissa: relative globule size.

A. Lectin

B. Lectin pre-incubated with SR glycopeptide

C. Lectin pre-incubated with PP glycopeptide

D. Lectin pre-incubated with desialylated SR glycopeptide.


A
C
B
D

Discussion

It is now well established that the milk fat globule membrane (MFGM) is derived primarily from the apical plamsa membrane of mammary alveolar epithelial cells (Patton and Keenan, 1975). While the MFGM has become increasingly popular as a model for studies of plasma membrane properties, most research has been concentrated on the extraction of proteins and glycoproteins by various methods from milk fat globule membranes. Sugar residues exposed on the surface of intact milk fat globules may well be subjected to differential extraction or to conformational changes during purification of glycoprotein fractions while glycolipid components are largely lost. It is clear that contribution of carbohydrate structures to specific interactions of the MFGM and, by implication, also the mammary epithelial cell membrane, can most directly be studied in the intact fat globule. Exposure of monosaccharide residues has accordingly been examined on the outer membrane surface of intact and neuraminidase treated bovine and human milk fat globules by making use of lectin-induced agglutination of milk fat globules and of the fluorescent-activated cell sorter (FACS).

Pooled bovine milk fat globules were found to be agglutinated by high dilutions of Ricinus communis (types I and II), soya bean and wheat germ agglutinins, concanavalin A (Con A) and Helix pomatia lectin. In all cases agglutination was specifically inhibited by the corresponding monosaccharide receptor. Specific agglutination by these lectins demonstrates the accessibility of galactose (Ricinus types I and II, soya bean), mannose and/or glucose (Con A),

N-acetyl-D-galactosamine (Helix pomatia) and N-acetyl-D-glucosamine (wheat germ agglutinin) on the surfaces of intact bovine milk fat globules. The degree of interaction of the fluorescent derivatives of the above lectins (with the exception of Con A) was assessed by the FACS and the results obtained were in general agreement with those obtained from the agglutination assay, a correlation which stresses the usefulness of the agglutination assay as a sensitive and reliable technique in studying surface receptors on milk fat globules. These observations are entirely consistent with the gas chromatographic evidence in that sialoglycopeptides isolated from the surface of bovine milk fat globules by proteolytic treatment contain all the above sugars together with sialic acid and fucose (Section A). Sialic acid was shown (Newman and Harrison, 1973) by microelectrophoretic technique to be exposed on the surface of intact bovine milk fat globules whereas other sugars were not detectable by these means. Although the presence of fucose in bovine MFGM-derived glycopeptides has been demonstrated by gas chromatography (Section A), this was not reflected in agglutination of the globules by either of the fucose-specific lectins, Ulex europaeus or Lotus tetragonolobus. Horisberger et al. (1977) were similarly unable to demonstrate marking of bovine milk fat globules by gold-labelled anti-H lectin and it appears that although fucose is normally a chain-terminal unit, access to the sugar is hindered not only in the case of gold-labelled lectins but also in that of the underivatized reagents.

In contrast to the findings of Horisberger et al. (1977), specific interaction of bovine milk fat globules with peanut agglutinin

(Arachis hypogaea) could be demonstrated by means of the agglutination assay and by the FACS. Peanut agglutinin has been shown (Lotan et al., 1975; Pereira et al., 1976) to have high affinity for the Thomsen-Fredenreich(TF) antigen (β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosamine) and the Arachis-induced agglutination of bovine milk fat globules was specifically inhibited by low concentrations of the free reduced TF antigen. Another piece of evidence supporting the above observations was provided by the fact that the desialylated SR glycopeptide, which is known (Farrar, 1978) to carry high concentrations of TF antigen, specifically inhibited the Arachis-induced agglutination of bovine globules, whereas both native and alkaline borohydride-treated SR glycopeptide showed no inhibitory activities. Sialic acid-substituted TF antigen has been shown to be released from the PP sialoglycopeptide (Farrar and Harrison, 1978) as well as from the SR glycopeptide (Farra, 1978) by alkaline borhydride treatment in the form of two trisaccharides and a tetrasaccharide fraction (Fig. 24). The unsubstituted TF antigen was not specifically sought in these studies but has been detected in a phenol-extracted glycoprotein fraction from bovine MFGM by Newman et al., (1976) who used a haemagglutination-inhibition technique. The present evidence for the exposure of unmasked TF antigen on the surface of bovine milk fat globules clearly complements these latter findings and indicates that the presence of unsubstituted disaccharide in extracted glycoproteins does not result from artefactual cleavage of sialic acid residues during the extraction procedure. The failure of Horisberger et al., (1977) to mark bovine fat globules with gold-labelled peanut lectin could reflect a more restricted access

of the bulky labelled reagent to the surface receptors.

As might be expected, pre-treatment of bovine milk fat globules with neuraminidase led to cleavage of sialic acid residues from the surface tri-and tetra-saccharide (Farrar, 1978; Farrar and Harrison,1978) with consequent exposure of the TF antigenic core. It is of interest that Vicia graminea which is known (Uhlenbruck and Dahr, 1971) to require the presence of TF antigen in its specific receptor site, only causes agglutination of bovine globules after neuraminidase treatment of the globules when the concentration of exposed disaccharide was increased.

The presence of N-acetyl-D-galactosamine in an exposed form on the surface of bovine milk fat globules was shown by the specific agglutinations induced by Helix pomatia and Dolichos biflorus lectins. This supports previous reports (Newman et al., 1976; Newman and Uhlenbruck, 1977) that phenol-extracted glycoproteins from bovine MFGM carry unsubstituted N-acetyl-D-galactosamine. This is also consistent with our findings that treatment of the SP glycopeptide with periodate (Section A) resulted in the destruction of all its N-acetyl-D-galactosamine indicating that this sugar is terminal in position. The agglutination titre was slightly increased by neuraminidase treatment of the bovine globules.

Human MFGM is less readily available than the bovine membrane and has been correspondingly less studied. Human milk fat globules were found to be specifically agglutinated by high dilutions of

Ricinus communis (types I and II), soya bean, concanavalin A, Lens and wheat germ agglutinins, demonstrating the accessibility of galactose (Ricinus, soya bean agglutinin)mannose and/ or glucose (Con A, Lens) and N-acetyl-D-glucosamine (wheat germ agglutinin) on the surfaces of intact fat globules. Agglutination of human globules by these lectins is generally similar to that of bovine globules although titres, particularly for Con A and Lens, were higher in the human case. As for bovine globules, agglutinations were specifically inhibited by the corresponding hapten sugars. These observations are entirely consistent with the gas chromatographic evidence (Safi, 1981) that sialoglycopeptides isolated from the surface of human milk fat globules by proteolytic treatment contain all the above sugars together with sialic acid and fucose. These findings also support the electron microscopic data of Horisberger et al. (1977) in which human milk fat globules were found to be marked by gold-labelled soya bean, wheat germ agglutinins and, although to a lesser extent, by Con A. The ability of intact human milk fat globules to bind soya bean agglutinin contrasts with the behaviour of extracted human MFGM glycoprotein components which interact weakly (Newman and Uhlenbruck, 1977) or not at all (Murray et al., 1979) with soya bean lectin. It may be that soya bean lectin-induced agglutination of intact human milk fat globules and marking of the globules by gold-labelled soya bean lectin (Horisberger et al., 1977) involves exclusively glycolipid-bound galactose. The increased interactions (Table 22 and Horisberger et al., 1977)observed following neuraminidase treatment of the globules suggest, however, that glycoprotein-bound galactose is involved at least in the

response of sialic acid-free globules to soya bean lectin because, as Horisberger et al.(1977) have pointed out, MFGM proteins mask membrane gangliosides from neuraminidase attack (Tomich et al., 1976).

As with bovine globules, human milk fat globules were not agglutinated by the fucose-specific lectins, Ulex europaeus and Lotus tetragonolobus although weak agglutination was detected with the latter lectin follwoing neuraminidase treatment of the human globules. Most lectins do, in fact, show increased agglutination titres after treatment either of bovine or human fat globules with neuraminidase. It is likely that such increases generally reflect unmasking of relevant sugar receptors as a consequence of the removal of terminal sialic acid-residues. It could be that enhanced agglutinability under such circumstances simply arises from non-specific aggregation resulting from loss of surface negative charges although the lack of increase of agglutination of human fat globules by Arachis following neuraminidase treatment of the globules, argue against this.

The human milk fat globules were also agglutinated by relatively high dilutions of the peanut agglutinin and as in the case of bovine fat globules, agglutination was specifically inhibited by the free (reduced) TF antigen and by the desialylated SR glycopeptide which is rich in the unsubstituted TF antigen. Neither the native SR glycopeptide nor the alkaline borohydride treated SR glycopeptide inhibited the Arachis-induced agglutination. The exposure of substituted TF antigen on the surface of intact

human milk fat globules has not previously been demonstrated, although Klein et al. (1978) have provided evidence for exposure of the antigen on the surface of normal mammary epithelial cells from which the globule membrane is derived. These results are contrary to those of Springer et al. (1975) who found the TF antigen to be exposed on breast carcinoma but not in normal tissue. Our results are also consistent with the findings of Newman et al. (1979) who found TF antigen in breast tissue cultures of normal individuals. As is the case with soya bean agglutinin, peanut lectin apparently behaves differently with extracted human MFGM glycoproteins. Thus, Newman and Uhlenbruck (1977) were unable to show inhibition of peanut lectin-induced agglutination of desialylated erythrocytes either by native or by desialylated human MFGM glycoprotines indicating that the TF antigen was not accessible on their glycoprotein extract, despite the fact that it was detected chemically in the same fraction (Glockner et al., 1976).

As mentioned above, human fat globules were not rendered more agglutinable by peanut lectin following their treatment with neuraminidase. This contrasts with the behaviour of bovine milk fat globules which carry appreciable amounts of sialylated TF antigen in tri- and tetrasaccharide forms (Farrar, 1978; Farrar and Harrison, 1978) exposed on their outer surface. These conclusions are supported by the results obtained using Vicia graminea lectin which failed to agglutinate human fat globules even after neuraminidase treatment of the globules. Under similar circumstances the TF antigens of bovine milk fat globules were apparently

unmasked sufficiently to allow agglutination by high dilutions of Vicia graminea lectin, even though, as in the human case, native globules were not agglutinated by highest concentrations of lectin. The above conclusions are suported by the findings of Safi (1981) that treatment of a major sialoglycopeptide isolated from intact human milk fat globules with alkaline borohydride resulted in the release of a tetrasaccharide fraction containing sialic acid, galactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine in the approximate molar ratios 3:3.5:2:1 respectively. TF antigen containing oligosaccharides were not reported in the human MFGM-derived sialoglycopeptides (Safi, 1981).

In contrast to bovine globules, human milk fat globules were not agglutinated by N-acetyl-D-galactosamine-specific lectins, Helix pomatia and Dolichos biflorus, irrespective of the blood group of the human donor. It is of interest that donors of A-blood group specificity express the A antigen (N-acetyl-D-galactosamine) on their red blood cells (by definition) but not apparently on milk fat globules. The opposite is true of the bovine system in which unsubstituted Helix pomatia receptor has been detected on intact milk fat globules but not on erythrocytes (Kim and Uhlenbruck, 1966). Results obtained from membrane-derived glycoprotein fractions agree with these findings in the bovine (Newman et al., 1977; Newman and Uhlenbruck, 1977) but not in the human case, when Helix pomatia receptors were detected on phenol-extracted glycoprotein fractions from human MFGM (Glockner et al., 1976). The above findings were, as in the case of bovine globules, assessed by the FACS and in all cases results were consistent with

thos-e obtained from the milk fat globule agglutination assay again stressing the validity of the latter assay.

The accessibility of carbohydrate residues in bovine MFGM-derived sialoglycopeptides to a range of fluorescent labelled lectins was studied by inhibition of fluorescein labelling of human globules by these lectins. Thus, the SP glycopeptide fraction inhibited labelling of the globules by Con A (mannose-specific) and wheat germ agglutinin (N-acetyl-D-glucosamine-specific). This is consistent with the gas chromatographic evidence that the SP glycopeptide is rich in mannose and N-acetyl-D-glucosamine (Section A) and suggests that these sugars are accessible to the above lectins. The SR glycopeptides, on the other hand, did not inhibit labelling of the globules by either Con A or wheat germ agglutinin whereas its desialylated and alkaline borohydride treated derivatives inhibited the labelling of globules by Con A but not by wheat germ agglutinin. This increased activity with respect to Con A could be due to unmasking of subterminal mannose residues following desialylation.

Sialic acid has been reported (Lis and Sharon, 1977; Goldstein and Hayes, 1978) to be important in the binding of wheat germ agglutinin to glycoprotein and glycopeptides but the present results do not support this. Agglutination titres of bovine and human globules by this lectin were not decreased following neuraminidase treatment of the globules and the SR glycopeptide failed to inhibit the interaction of this lectin with the human globules.

In spite of the gas chromatographic evidence that the SR glycopeptide is rich in galactose, it did not inhibit interaction of Ricinus II with the globules. This is consistent with the fact that most of the galactose is internal in position as part of the sialic acid-substituted TF antigen with the fact that only 30% of total galactose was destroyed by periodate treatment (Section A). Indeed, desialylation and alkaline borohydride treatment of the SR glycopeptide increased this inhibition to 50%. Galactose residues on the SP glycopeptide were similarly inaccessible to Ricinus-II. This was as this glycopeptide fraction is poor in galactose and the sugar could be totally masked by sialic acid. Galactose residues on all glycopeptides tested (Table 23), on the other hand, were accessible to soya bean agglutinin. These observations are in agreement with those of Murray et al. (1979) who showed that soya bean agglutinin was able to bind all seven glycoproteins isolated from bovine MFGM.

The milk fat globule agglutination assay employed in this study is a simple technique capable of rapidly providing quantitative information about the accessibility of a wide range of membrane-bound sugar components without the need for chemical derivatization. The general agreement of results obtained from this method and from the highly advanced FACS implies that the agglutination assay, besides being rapid and easy to perform is highly sensitive. The method is applicable to surface antigens in general and has been used to define immunodominant groupings (Section A) and receptors for bacteria (Section C) on the surfaces of bovine MFGM. The occasional differences between the agglutination

assay and FACS on the one hand and the elegant but more limited studies of Horisberger et al. (1977) using gold-labelled lectins, on the other, can be explained in terms of hindered access of the relatively-bulky gold-labelled lectins to the globule surface. Differences between interactions of lectins with intact globules on the one hand and with isolated glycoproteins on the other, are more marked and could well reflect changes resulting from extraction in the latter case. It may also be that glycolipids contribute significantly to the specific interactions of the MFGM surface.

One of the most significant differences between human and bovine MFGM show by agglutination and FACS studies is the lack of effect of neuraminidase on peanut lectin interaction with human globules suggesting that the latter do not carry significant amounts of tri- and tetrasaccharide residues which have been isolated from bovine globules (Farrar, 1978; Farrar and Harrison, 1978). These oligosaccharides have been characterized in membranes of human erythrocytes (Thomas and Winzler, 1969), porcine erythrocytes (Dukov, 1980), rat brain (Finne, 1975) and on other membranes (Krusius and Finne, 1977) and their apparent absence from human milk fat globules raises the problem of their function generally. A second major difference concerns the presence of Helix pomatia receptors on bovine but not on human milk fat globules despite the A-blood group specificity of many of the human donors. It is an intriguing fact that in both these cases the structure present on bovine but not on human globules has been reported in human but not in bovine erythrocyte membranes (Kim and Uhlenbruck, 1966; Thomas and Winzler, 1969; Emerson and Kornfeld, 1976).

The contribution of MFGM lectin receptors to specific interactions of the milk fat globule, or more significantly, of the mammary epithelial cell membrane, remain to be elucidated, but it is likely that these exposed sugar residues will be shown to play an important role in recognition processes involving mammary cells or epithelial cells generally.

Section C

Investigation of exposed receptors for the K99 adhesin on the surfaces of bovine milk fat globules and sheep erythrocytes

Introduction

The mucous membranes of mammals are the major routes of entry for microbial infections, and infections which are localized in, of invade via, the gastrointestinal tract are important causes of morbidity and mortality. Gastrointestinal disorders are especially common with many deaths occurring from Escherichia coli (E. coli) infection (Sojka, 1973) and from transmissible gastroenteritis. The latter has a mortality approaching 100% in pigs under 7 days of age although older pigs are not seriously affected (Bohl, 1973).

Metchinikoff (1905) was one of the first to consider the disturbing implication of man's lifelong coexistence with the huge numbers of potentially unpleasant organisms within him. Now, the immunological relationship of host to pathogen in the intestine is better understood, but still many questions remain concerning the nature of the usually peaceful relationship between the animal host and its normal enteric flora. The major property of the immune system is its ability to distinguish with great specificity between 'self' and 'non-self' organisms or substances. In the gastrointestinal tract, however, the immune system has evolved alongside the development of powerful and specific systems which allow totally separate organisms (such as man and E. coli or bacteroids) to maintain close symbiotic relationships. Thus, the immunological system of the gastrointestinal mucosa must have evolved the ability to distinguish not merely between self and non-self antigens, but also between 'acceptable' and 'non-acceptable' microorganisms within the intestine. It thereby preserves the ability to mount a defensive response against pathogens, and avoids the production of an appropriate response against the normal microflora (McCelland, 1979). Besides the adaptive immunological mechanisms of the intestinal immune system,

non-immune factors including diet, gastric acid secretion, intestinal mobility, anatomical disturbance, bile salt concentration, pH, redox potential as well as the interactions of various members of the intestinal microflora with each other and with the host (Savage, 1972; Jones and Rutter, 1972) are also involved. The overall stability of the intestinal flora is dependent upon the complex interactions between the above factors (Fig. 58).

Certain enteropathogenic strains of E. coli are associated with diarrhoeal diseases in pigs, calves, lambs and humans. Despite the differences between strains isolated from different animal species there are two general requirements for the production of disease; firstly successful binding of the organism and colonisation of the intestine and secondly release of heat-labile or heat-stable enterotoxins. The heat-labile enterotoxin of E. coli resemble cholera enterotoxin and activates adenylate cyclase with a resultant increase in cAMP and inhibition of sodium absorption. Thus, sodium no longer acts as a driving force and chloride is actively moved from blood to lumen, pulling cations and water with it into the gut lument to maintain the osmotic equilibrium with blood plasma (Fig. 59). (Finkelstein, 1976; Du Pont and Pickering, 1980).

The first clear evidence of the significance of bacterial adhesion in expressing pathogenicity came in the early 1970s when Smith and Linggood (1971) reported that the virulence of E. coli for early-weaned piglets increased after the bacteria had acquired the ability to produce K88 adhesin (antigen). This was followed by the report of Jones and Rutter (1972) in which they demonstrated

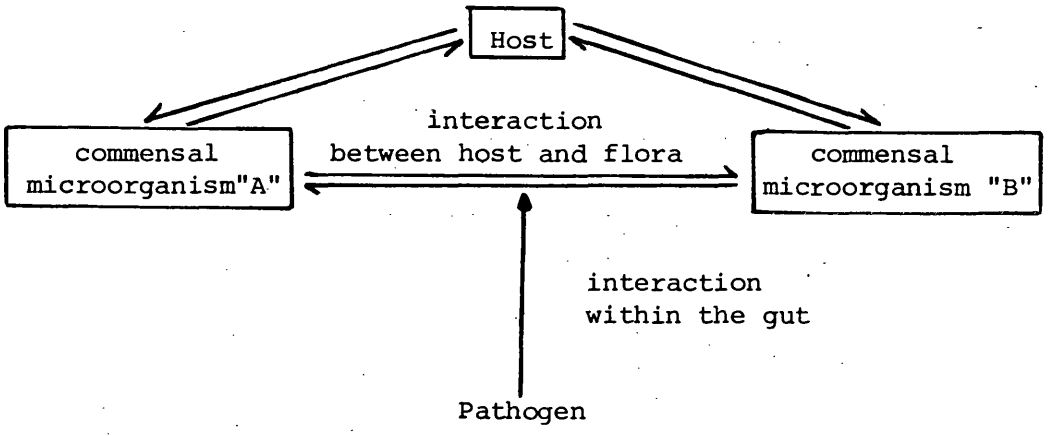


Fig. 58. The normal bacterial flora of the intestine are determined by complex interactions of bacteria with each other and with host's defence mechanisms. A pathogen must be able to disturb these relationships to establish itself and produce disease (After McClelland, 1979).

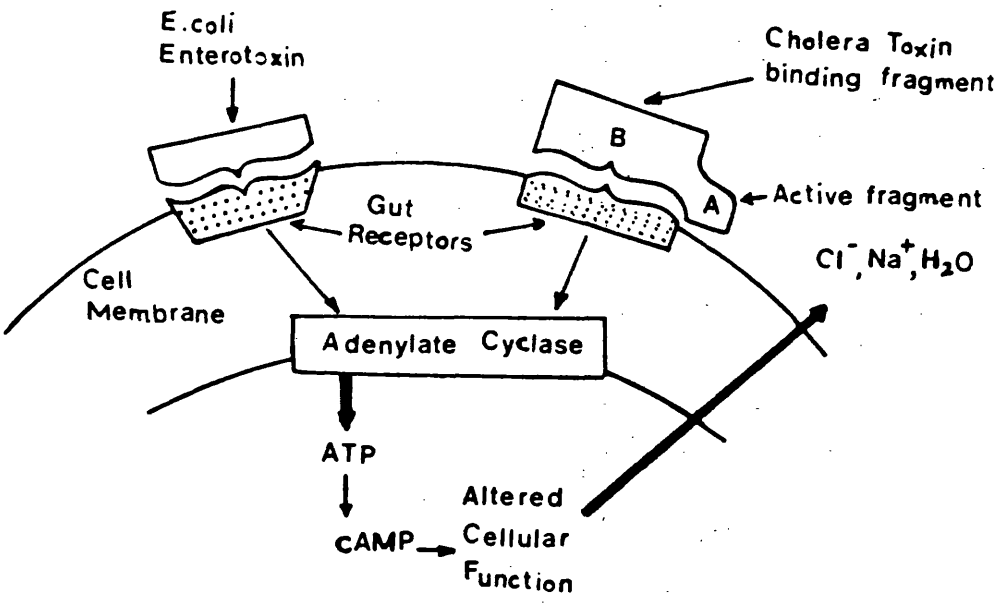


Fig. 59 Schematic representation of intestinal binding of cholera toxin and heat-labile E. coli toxin leading to intestinal secretion of fluid and electrolytes (After Du Pont and Pic-Kering, 1980).

that the K88 adhesin was synthesized by a K88-positive enteropathogenic E. coli (EPEC) in the upper intestine of both gonobiotic and conventional neonatal piglets where it functioned as an adhesive agent enabling the bacteria to adhere to and colonize the intestinal mucosa. K88-negative mutant strains, on the other hand, did not adhere to and colonize the mucosa. More recently, Rutter et al. (1976) reported that piglets suckled by dams vaccinated with K88 vaccine are not susceptible to challenge with K88-positive EPEC.

Another adhesin, referred to as K_{CO} adhesin, was reported to be present in enteropathogenic strains in calves and lambs. (Smith and Linggood, 1972). The adhesin is now known as K99 adhesin (Ørskov et al., 1975) and has since been found on some atypical strains of E. coli isolated from piglet neonatal diarrhoea (Moon et al., 1977). Burrows et al. (1976) concluded that the K99 adhesin is responsible for both adhesive and haemagglutinating (of sheep erythrocytes) properties of K99 positive E. coli strains. Both the K88 (Smith and Linggood, 1971) and the K99 (Smith and Linggood, 1972) adhesins are governed by transmissible extrachromosomal genes or plasmids. K99 adhesin isolated by acetate precipitation from E. coli B41 (the established K99 reference strain) was found to be anionic with pI of 4.2 and to agglutinate guinea pig and sheep erythrocytes (Morris et al., 1978a). Similarly, Burrows et al. (1976) reported that the K99 adhesin isolated from a K12 ($K99^+$) recombinant by ammonium sulphate precipitation was cationic with a pI of 10 and failed to agglutinate erythrocytes from either guinea pigs (Isaacson, 1977) or sheep (Morris et al., 1978a). Subsequent work by Morris et al. (1978b) showed that cell-free K99 adhesin from the reference strain B41 is composed of two antigenically distinct components, an

anionic component with stable haemagglutinating activity and a cationic component with a labile haemagglutination reaction. More recently, it was shown that both components adhere to calf brush borders (Morris et al., 1980a). As with the case of piglets, lambs can be passively protected from diarrhoea after oral challenge with a virulent enteropathogen by ingestion of colostrum from dams vaccinated with K99 vaccine (Sojka et al., 1978). This was attributed to the neutralisation of the adhesive properties of the K99 positive E. coli by colostral antibodies (Morris et al,, 1980b) The general characteristics of the K88 and K99 adhesins are shown in Table 25. More details about bacterial adhesion can be found in a number of reviews (Carpenter, 1972; McClelland, 1979; Porter, 1979; Arbuthnott and Smyth, 1979; Du Pont and Pickering, 1980).

Reiter and Brown (1976) briefly reported that bovine milk fat globule membranes have the ability to inhibit the agglutination by K99 adhesin of sheep erythrocytes. In view of the availability of surface glycopeptides of bovine milk fat globule membrane (MFGM) it was decided to investigate their possible involvement in the interaction of the K99 adhesin with the brush border membrane, the suggestion being that certain membrane components might be common to bovine epithelial membranes. In the first instance the interaction of K99 adhesin with MFGM components was investigated by using the agglutination assay described in the previous section.

Table 25. Adhesins associated with enteropathogenic *E. coli* strains (after Arbuthnott and Smyth, 1979)

Characteristic	K88 Antigen	K99 Antigen	Colonisation Factor	Adherence Factor
Origin of strains causing diarrhoea	Piglets	Calves, Lambs	Man	Man
Genetic control	Plasmid	Plasmid	Plasmid	Plasmid
Electron microscopic morphology	Pilus-like layer of flexible filaments	Pilus-like, individual fibrous rods, mean length 120nm, mean dia.8.4nm	Pilus-like	?(Lipo)poly-saccharide
Chemical nature	Protein, 4% lipid	Protein, 6.6% lipid, 0.6% carbo-hydrate	?	?
Physical properties:				
Sedimentation coefficient(S_{20_w})	36.7S	13-15S	?	?
Molecular weight of SDS subunits	23,000	22,500 and 29,500	?	?
Isoelectric point	?	10.0	?	?
Haemagglutinating properties:				
Erythrocyte species	Guinea-pig	Sheep	Human	
Inhibition by monosaccharides	Mannose-insensitive	Mannose-insensitive	Mannose-insensitive	
Absence on bacteria grown at 18°	+	+	?	?
Heat-lability	+	+	+	?
Adhesive properties for intestinal brush borders	(piglet) +	(calf) +	(rabbit) +	(human foetal) +

Materials and Methods

I. Materials

K99 adhesin from *E. coli* reference strain B41 was kindly supplied by Dr. J.A. Morris, Central Veterinary Laboratory, Weybridge, Surrey, U.K. Fucose, mannose, galactose, glucose, N-acetyl-glucosamine and N-acetylgalactosamine were all obtained from Sigma Chemical Co., London, SW6. Sephadex gels were from Pharmacia Ltd., London W5. Phosphate buffered saline (PBS) tablets were obtained from Oxoid Ltd., Basingstoke, Hants, U.K. Microtiter plates were obtained from Sterilin Ltd., Teddington, U.K. Anti A and Anti B antibodies were generous gifts from Dr. D.J. Anstee, South West Regional Blood Transfusion Center, Southmead, Bristol.

Fresh bovine milk was obtained from Friesian cows in mid-lactation and each sample was a pool from four individual animals. Human milk was from individual volunteers in established lactation.

Human blood type A and B was from individual volunteers, University Medical Centre. Sheep blood was obtained from Flow Laboratories, England, and calf blood from Tissue Culture Services, England.

All other reagents were from BDH Chemicals Ltd., Poole, Dorset, U.K.

II. Methods

Milk fat globule agglutination assay

The milk fat globule agglutination assay was carried out using the K99 antigen as described for lectins in the previous section. Inhibition studies were carried out by using free sugars and bovine MFGM-derived sialoglycopeptides. The inhibitor was serially diluted (2x) and incubated at 26°C for 15 min with four agglutinating doses of the K99 antigen followed by addition of globule suspension prepared as described in the previous section. The starting concentrations of the inhibitors were 1 M in the case of free sugars and 8 mg/ml in the case of sialoglycopeptides. Agglutination titres are expressed as the reciprocal of the minimum dilution of the K99 antigen giving agglutination of less than 30% of the total fat globules. Inhibition titres are expressed as the minimum concentration of the inhibitor required to inhibit four agglutination doses of the K99 antigen.

Haemagglutination assays

Blood samples were washed and 3% cell suspensions were prepared as described in the previous section. For the haemagglutination assays, test cells (50 µl of 3% suspension) were mixed with K99 antigen (50 µl) and allowed to stand in the well of a microtitre plate for 1 h at 26°C. Absence of agglutination was indicated by the presence of a compact 'button' of cells at the bottom of the well. Inhibition studies were carried out by preincubating four agglutination doses of the K99 adhesin with inhibitor-containing buffer for 15 min at 26°C followed by addition of the cell suspension. Inhibition titres are expressed as stated for the globule assay.

Bovine MFGM-derived sialoglycopeptides

Sialoglycopeptides were cleaved off intact washed bovine milk fat globules by treatment with pronase as described in Section A. The pronase digested membrane materials were fractionated on Sephadex G-50 to give a major peak which, when fractionated by ion-exchange chromatography gave two fractions SP and SR (Section A). The SR glycopeptide fraction was shown to contain high concentrations of galactose and N-acetylgalactosamine whereas the SP fraction was shown to contain high concentrations of mannose and N-acetylglucosamine.

Chemical and enzymic modifications of the sialoglycopeptides were carried out as described in Section A.

Gas liquid chromatography

Quantiative estimation of individual sugars in the K99 adhesin as their alditol acetate derivatives was carried out by gas liquid chromatography as described in Section A for the glycopeptides. Samples containing 2.4 mg protein of the adhesin were derivatized and aliquots (1 µl containing 120 µg protein) were injected onto columns (2 m x 0.2 cm) of coiled glass containing 3% OV-225 on H.P. Gas Chrom Q. Chromatography was done isothermally at 200°C with a flow rate of 45 ml/min. The instrument was a Perkin-Elmer Sigma 3 gas chromatograph equipped with dual flame ionization detectors.

Results

Agglutination by K99 adhesin of intact cells and fat globules

K99 adhesin was found to agglutinate sheep red blood cells with a titre of 2^{7-8}, in accordance with the presently accepted use of this system as a measure of K99 adhesin activity (Burrow et al., 1976; Morris et al., 1980 a).

Washed bovine milk fat globules were also agglutinated by similarly high dilutions of K99 adhesin, suggesting that bovine intestinal cell membrane receptors for the adhesin are also present on bovine milk fat globules. In accord with the demonstrated species specificity of the pathogenicity of the parent K99-bearing strain of E. coli, human milk fat globules were not agglutinated by K99 adhesin.

In contrast to bovine milk fat globules, calf red blood cells were not agglutinated by K99 adhesin. Human erythrocytes were, on the other hand, agglutinated, type A cells being more strongly affected than type B cells.

The agglutination titres are listed in Table 26.

Inhibition of agglutination of bovine MFG by glycopeptides of bovine MFGM origin

Sialoglycopeptides were cleaved from intact bovine milk fat globules as described in Section A and partially purified by gel filtration on Sephadex G-50. Material corresponding to the major hexose-sialic acid peak (Fig. 25) was freeze-dried and tested as an

	Agglutination titres
Sheep blood cells	2^{7-8}
Bovine milk fat globules	2^{4-5}
Human milk fat globules	-ve
Calf blood cells	-ve
Human type A cells	2^{7-8}
Human type B cells	2^{4-5}

Table 26. Agglutination titres of a range of blood cells and milk fat globules by K99 adhesin. Titres are expressed as the reciprocal of the minimum dilution of the K99 giving no agglutination.

inhibitor of agglutination of bovine milk fat globules as described in the Materials and Methods section. This material, referred to as PP (partially purified) sialoglycopeptide, showed an inhibition titre of 1.3 mg/ml (Table 27).

Further fractionation of PP-sialoglycopeptide by ion-exchange chromatography on DEAE Sepharose CL6B allowed separation of a sialic poor (SP) and a sialic acid rich (SR) glycopeptide as described in Section A (pp. 72-76). Both of these glyccpeptides showed increased inhibitory activity in the bovine fat globule agglutination assay relative to that of the precursor (PP glycopeptide) but the activity of the SR glycopeptide was particularly strong (Table 27).

In order to try and determine the relative contributions of the various components of the sialoglycopeptide molecules to their inhibitory activity, both SR and SP glycopeptides were systematically modified in a number of ways and the modified glycopeptides were examined as inhibitors of the agglutination assay. Thus desialylation of either SR or SP sialoglycopeptides increased the inhibitory activity by a factor of 2.3 fold showing that the activity was not simply a non-specific anionic effect (Table 27). In order to assess the contribution made by the carbohydrate components of the glycopeptides to their inhibitory activity, both SR and SP glycopeptides were treated with periodate (Section A, pp. 93,103)and reassayed. Again the result was the same in both cases but this time showed complete abolition of inhibitory activity suggesting the importance of the carbohydrate in this respect. This conclusion was supported by further experiments in which approximately 50% of the carbohydrate

Inhibitor	Inhibition titre
PP sialoglycopeptide	1.30 mg/ml
Alkaline borohydride treated PP	2.60 mg/ml
SR glycopeptide	0.08 mg/ml
Desialylated SR	0.04 mg/ml
Periodate oxidized SR	-ve
T. Foetus treated SR	-ve
Alkaline borohydride treated SR	0.04 mg/ml
SP glycopeptide	0.66 mg/ml
Desialylated SP	0.2 mg/ml
Periodate oxidized SP	-ve
T. Foetus treated SP	-ve
L-Fucose	-ve
D-Mannose	-ve
D-galactose	-ve
D-Glucose	-ve
N-Acetyl-D-glucosamine	-ve
N-Acetyl-D-galactosamine	0.5 M
TF antigen	-ve

Table 27. Inhibition titres of K99-induced agglutination of bovine MFGs by MFGM-derived sialoglycopeptides and a range of free monosaccharides. Titres are expressed as the minimum concentration of the inhibitor (in the final volume) required to inhibit the agglutination of bovine MFGs by four agglutination doses of the K99 adhesin.

residues of both SR and SP glycopeptides were removed by a mixture of glycosidases from Trichomonas foetus (Section A, pp. 97, 107) and the treated glycopeptides failed completely to inhibit the agglutination of bovine milk fat globules (Table 27). In contrast to these findings, however, treatment of the SR glycopeptide with alkaline borohydride, designed to remove O-glycosidically-linked oligosaccharide residues from the glycopeptide slightly increased its inhibitory activity. Corresponding treatment of PP-glycopeptide, on the other hand, led to a two fold decrease in inhibition potency (Table 27).

In view of the evidence, obtained from periodate and glycosidase treatment, of the importance of carbohydrate residues to the ability of the MFGM sialoglycopeptides to inhibit agglutination, the effect of various monosaccharides on the agglutination assay was investigated. As can be seen from Table 27, only N-acetylgalactosamine showed any ability to inhibit the agglutination of bovine milk fat globules and even this sugar was only effective at relatively high concentrations.

The Thomsen-Friedenreich, β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosamine, is known (Section A) to be a major component of the SR glycopeptide and the antigen, or more accurately, its reduced form, β-D-galactopyranosyl-(1→3)N-acetyl-D-galactosaminitol was examined as a potential inhibitor in the agglutination assay. No inhibition could be demonstrated at the levels of the disaccharide (0.33 mg/ml) tested.

Inhibition of agglutination of sheep erythrocytes by glycopeptides from MFGM

The modified sialoglycopeptide fractions from bovine MFGM were tested for their ability to inhibit the agglutination of sheep red blood cells. Inhibitions were in all cases less powerful than in the corresponding case for bovine milk fat globules but the general tendencies were the same (Table 28). Thus inhibitory activity was slightly increased by desialylation or alkaline borohydride treatment of the glycopeptide and completely destroyed by corresponding treatment with periodate or glycosidases. N-acetylgalactosamine, alone among the monosaccharides tested, was capable of inhbiting the agglutination of erythrocytes and this required relatively high concentrations of the sugar (0.5 M).

Carbohydrate composition of the K99 adhesin

The purified K99 adhesin was subjected to hydrolysis and carbohydrate analysis exactly as described in Section A for milk fat globule-derived sialoglycopeptides. The results of gas chromatographic analysis of individual monosaccharide components are shown in Table 29 and the chromatographic trace is shown in Fig. 60.

Inhibitor	Inhibition titre
PP Sialoglycopeptide	2.0 mg/ml
Alkaline borohydride treated PP	4.0 mg/ml
SR glycopeptide	2.0 mg/ml
Desialylated SR	1.0 mg/ml
Periodate oxidized SR	-ve
T. Foetus treated SR	-ve
Alkaline borohydride treated SR	1.0 mg/ml
SP Glycopeptide	>10.0 mg/ml
Desialylated SP	8.0 mg/ml
Periodate oxidized SP	-ve
T. Foetus treated SP	-ve
L-Fucose	-ve
D-Mannose	-ve
D-Galactose	-ve
D-Glucose	-ve
N-acetyl-D-glucosamine	-ve
N-Acetyl-D-galactosamine	0.5 M
TF antigen	-ve

Table 28. Inhibition titres of K99-induced agglutination of sheep erythrocytes by MFGM-derived sialoglycopeptides and a range of monosaccharides. Titres are expressed as the minimum concentration of the inhibitor required to inhibit the agglutination of sheep erythrocytes by four agglutination doses of the K99 adhesin.

Monosaccharide	Content mg/100 mg protein
L-Fucose	-
D-Mannose	0.30
D-Galactose	0.54
D-Glucose	-
N-acetyl-D-glucosamine	0.40
N-acetyl-D-galactosamine	0.20

Table 29. Carbohydrate composition of the K99 adhesin

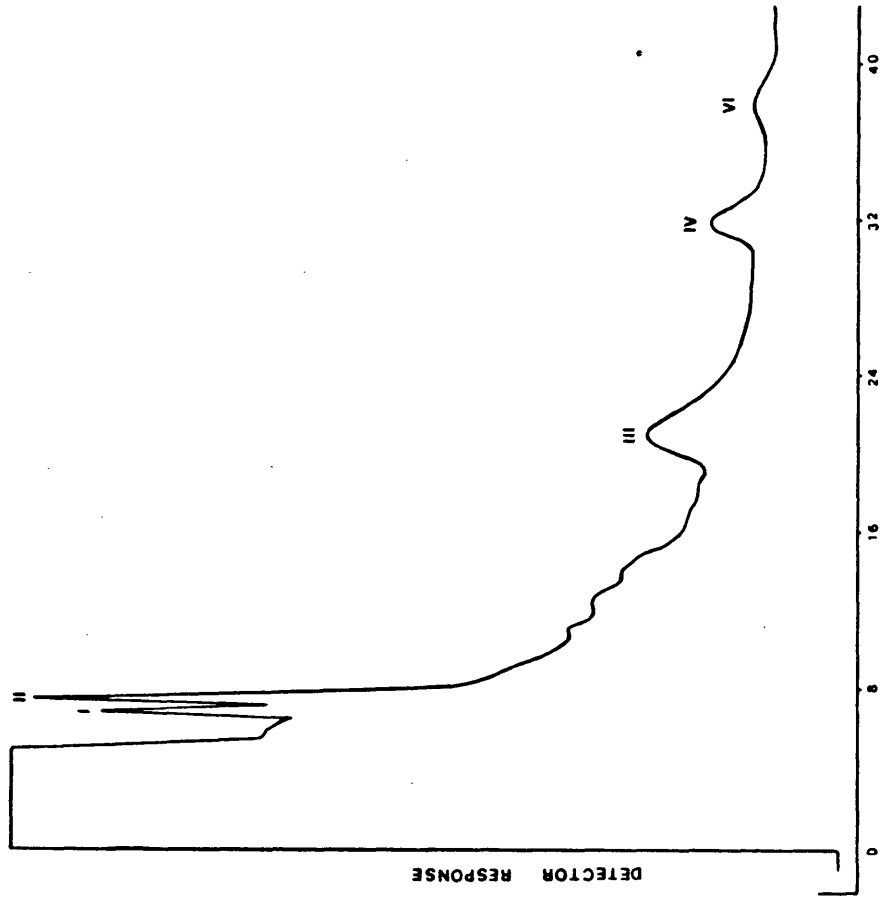


Fig. 60: Gas chromatogram of alditol acetate derivatised monosaccharides released during acid hydrolysis of the K99 adhesin. Peaks I-VI correspond to: D-mannose, D-galactose, Perseitol (internal standard), N-acetyl-D-glucosamine and N-acetyl-D-galactosamine respectively. Chromatography was performed isothermally at 200°C on a column of 3% OV-225.

Discussion

The ability of certain bacteria to adhere to biological membranes is recognised as being a fundamental requirement for colonisation of the host *in vivo*. With pathogenic organisms adhesive mechanisms can be of paramount importance for these strains to be established in preference to normal commensals. In particular this phenomenon plays an important role in the pathogenesis of enteric infections and dental plaque. While it is clear that adhesion is an important factor in pathogenicity the biochemistry of the process in general is poorly understood. A clear understanding of the biochemical basis of adherence requires knowledge of the surface components on the pathogen responsible for adherence and knowledge of the corresponding receptor(s) on the tissue cells of the host. Model systems may play a valuable role in defining such receptors and consequently the mechanisms involved in the adhesion process. In this respect haemagglutination techniques can often prove useful and, indeed, an association of adhesion and haemagglutinating properties is now well recognised in many species of Enterobacteriaceae (Duguid *et al.*, 1955; Duguid and Gillies, 1958). Thus, the ability of the K88 adhesin of procine enteropathogenic *E. coli* to effect haemagglutination of guinea pig erythrocytes has been used in detection (Jones and Rutter, 1974) and in correlation of the adhesin with adhesion. (Gibbons *et al.*, 1975). More recently, Parry and Porter (1978) showed that chicken erythrocytes can provide a useful model for defining the underlying mechanisms of interaction between K88 adhesin-bearing *E. coli* and host cell membranes. Burrow *et al.* (1976), on the other hand, showed that K99 adhesin activity is readily demonstrated by haemagglutination of sheep erythrocytes and brush border attachment tests. This was later confirmed by

Morris et al.(1980a) who showed that the anionic component of the K99 adhesin which is able to adhere to calf brush borders also agglutinated sheep, guinea pig and horse erythrocytes. The cationic component, on the other hand, failed to adhere to calf brush borders and also to agglutinate sheep erythrocytes (Isaacsson, 1978; Morris et al., 1980a) but was able to agglutinate horse erythrocytes (Morris et al., 1980a). From these results it was concluded that the adhesin(s) for calf brush borders are carried on the structure that haemagglutinates sheep erythrocytes.

Reiter and Brown (1976) have briefly reported that the agglutination of sheep erythrocytes by the K99 adhesin could be inhibited by bovine MFGM preparations. Following this report and in view of the establishment of the milk fat globule agglutination assay (Section B), it was decided to investigate the use of bovine MFGM as a model system, in which to study the interaction of the K99 adhesin with intestinal brush border membranes.

Bovine milk fat globules were found to be agglutinated by relatively high dilutions of K99 adhesin while human milk fat globules were not agglutinated even by undiluted adhesin. In view of the fact that the K99-bearing strain of E. coli (B41) is pathogenic to calves but not to humans, this suggests that the specific receptor for the adhesin is, as hoped, common to and specific for the epithelial membranes of the intestinal and mammary cells of the cow. The specificity of agglutination was further demonstrated by the inability of K99 adhesin to agglutinate calf erythrocytes. Insofar as erythrocytes and milk fat globules of particular species

have been systematically compared with regard to their surface properties, it has been found that there is little similarity to be found in the glycopeptide components of the two membranes (Newman et al., 1976a,b; Section B, pp. 182-193). Further evidence of the differences between fat globules and erythrocytes from the same species was provided by the observation that, unlike human globules, human erythrocytes and particularly type A cells were readily agglutinated by relatively high dilutions of the K99 adhesin. This supports the above arguments and raises the possibility that N-acetylgalactosamine is important in receptor binding sites for the K99 adhesin.

In an approach to defining the molecular nature of the receptor(s) for K99 adhesin on the surface of bovine milk fat globules experiments were carried out in which the agglutination of bovine milk fat globules by K99 adhesin was inhibited by MFGM surface components. Thus, agglutination was found to be strongly inhibited by a crude sialoglycopeptide fraction (PP) cleaved by proteolytic enzymes from bovine milk fat globules suggesting that the PP sialoglycopeptide carries at least part of the K99 adhesin receptor(s). When the PP sialoglycopeptide was further fractionated by ion-exchange chromatography the resulting fragments (SP and SR glycopeptides) showed increased inhibitory activity in the bovine milk fat globule agglutination assay relative to that of the precursor glycopeptide. The activity of the SR glycopeptide was particularly strong with an end point of 0.08 mg/ml which represents over sixteen fold increase following ion-exchange. These results strongly suggest that the major receptors for K99 adhesin on the surfaces of bovine milk fat globules are carried on the SR glycopeptide

fraction. It initially seemed possible that the inhibitory activity of the SP and SR glycopeptides might simply reflect the presence of negatively-charged sialic acid units exerting a non-specific effect. In order to examine this possibility the sialoglycopeptides were individually desialylated by treatment with dilute acid and then retested as inhibitors in the agglutination assay. Far from being decreased, the inhibition actually increased following desialylation indicating that the sialic acid residues were not the important factors in the inhibitory effect. A similar conclusion can be drawn from the observation that inhibitory activity also increased when the SR glycopeptide was treated with alkaline borohydride. Alkaline borohydride is known to remove oligosaccharides rich in sialic acid (SectionA) and it appears that the sialic acid residues might be acting to shield the true receptors from interacting with the adhesin.

Treatment of either SR or SP glycopeptide with periodate resulted in the abolition of their inhibitory activity suggesting that the carbohydrate residues on the glycopeptides are important in their interaction with the K99 adhesin. It is possible, however, that loss of activity as a result of periodate treatment of the glycopeptides could have resulted from the oxidation of amino acids, particularly hydroxy amino acids, in the glycopeptide chains. Spiro (1964) has stressed that the molar ratio of periodate:glycoprotein is critical if the specific destruction of carbohydrate is to be ensured and recommended ratios of 100:1 and 300:1 for this purpose. Ratios in excess of these values could lead to oxidation of protein residues. In our experiments the ratio of periodate:glycopeptide was

within these limits (200:1) and the loss of activity of the periodate-treated SR and SP glycopeptides can most probably be attributed solely to the destruction of carbohydrate residues.

In order to further examine this conclusion both SR and SP glycopeptides were treated with mixed glycosidases from T. foetus before being examined as inhibitors of the agglutination assay. Neither the SR glycopeptide nor the SP glycopeptide retained its inhibitory activity in the bovine milk fat globule agglutination assay following exposure to glycosidases. These observations strongly support the idea that carbohydrates are important in the interactions of the K99 adhesin with the bovine MFGM-derived sialoglycopeptides and by implication with bovine MFGM itself. As a check on the extent of deglycosylation brought about by the glycosidase mixture, the residual polypeptides were analysed by gas-liquid chromatography when it was found that approximately 42% and 78% of total carbohydrate had been removed from the SR and SP glycopeptides respectively. It has been shown by others (Watkins, 1966a; Westwood et al., 1976) that the T. foetus mixture is free from protease activity.

Inhibition studies used the particular carbohydrate residues that are involved in the binding of the K99 adhesin to bovine MFGM. Of these monosaccharides only N-acetylgalactosamine was able to specifically inhibit the agglutination of bovine milk fat globules by the K99 adhesin. Even in this case relatively high concentrations (0.5 M) was required before an effect was observed. The inhibitory activity of N-acetylgalactosamine helps to explain the fact that the K99 adhesin reacted more strongly with human type A erythrocytes

than with type B erythrocytes and supports the finding that the SR glycopeptide, which has high levels of N-acetylgalactosamine, was the most active glycopeptide in the inhibition assay. Furthermore, the failure of the K99 adhesin to interact with the human milk fat globules might well be partially explained by their lacking exposed N-acetylgalactosamine on their surfaces (Section B).

As N-actylgalactosamine is part of the Thomsen-Friedenreich (TF) antigen (β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosamine) and in view of the presence of relatively high levels of the substituted TF antigen in the SR glycopeptide (Section A) it was thought that the K99 adhesin might actually have specificity for the TF antigen. As shown in SectionA, treatment of the SR glycoptptide with alkaline borohydride resulted in the release of the tetrasaccharide N-acetylneuraminyl-(2→3)-β-D-galactopyranosyl-(1→3)-[N-acetylneuraminyl-(2→6)] -N-acetyl-D-galactosamininitol. Mild acid hydrolysis (0.2 N H_2SO_4, 80°C, 1h) of this tetrasaccharide followed by ion-exchange chromatography on DEAE-Sepharose CL 6B resulted in the isolation of the free reduced TF antigen (β-D-galactopyranosyl-(1→3)-N-acetyl-D-galactosaminitol). Experiments using the reduced TF antigen at a concentration of 0.33 mg/ml showed no inhibitory activity. In fact the failure of the TF antigen to interact with the K99 adhesin might with hindsight, have been predicted from the fact that the K99 adhesin failed to agglutinate human milk fat globules, which were shown (Section B) to have high levels of the unsubstituted (free) TF antigen exposed on their surfaces. However,higher concentrations of the TF antigen should be used in the assay in order to confirm the above conclusion.

These concentrations were in practice limited by shortage of material. The results of alkaline borohydride treatment of the sialoglycopeptide are not clear cut. Alkaline borohydride treatment of glycoproteins releases the alkali-labile oligosaccharide chains which are O-glycosidically-linked to serine or threonine via N-acetylgalactosamine residues. Thus, one might expect that treatment with alkaline borohydride would result in the release of all N-acetylgalactosamine of the glycopeptide as this sugar is usually confined to O-glycosidically-linked complexes. However, gas liquid chromatography of the residual glycopeptide showed that only approximately 53%of the N-acetylgalactosamine was released by these means. The slight increase in inhibitory activity of the SR glycopeptide following alkaline borohydride treatment matches that brought about by simple desialylation and can perhaps best be explained in terms of unmasking of an underlying (galactosamine-containing) carbohydrate structure.

From the data discussed above it can be concluded that carbohydrate complexes - containing N-acetylgalactosamine are the most likely candidates as the K99 receptors on the surfaces of bovine MFGM and by extension also the intestinal epithelial membrane.

Similar inhibition experiments carried out with agglutination of sheep erythrocytes rather than bovine milk fat globules gave essentially parallel results. Thus,the MFGM-derived sialoglycopeptides and their modified derivatives inhibited agglutination according to the same pattern although the inhibitory activities were less in the case of sheep erythrocytes. The parallel results from bovine milk fat globule and sheep erythrocyte agglutination

assays stresses the point that the bovine milk fat globule assay is, indeed, a valid assay to demonstrate the activity of the K99 adhesin *in vitro*. The use of milk fat globules in general as a model system for epithelial membranes is of considerable potential importance although it is premature to draw decisive conclusions as more detailed studies are needed to establish possible correlations between different adhesins and milk fat globule membranes of their respective hosts as well as other susceptible animals. Such studies may well lead to the establishment of the MFGM as a tool in studying the biochemical basis of the adhesion process.

The chemical nature of the K99 adhesin is not clear. Morris *et al.* (1977) concluded that the K99 adhesin of *E. coli* B41 is a glycoprotein with a terminal N-acetylgalactosamine moiety which is responsible for or involved in the K99 haemagglutination activity i.e. receptors for this sugar are present on the external surface of sheep erythrocytes. This conclusion was based on two observations firstly the loss of K99 adhesin activity following its treatment with periodate and secondly the great reduction (by 132 haemagglutination units) of the K99 haemagglutinating activity following its absorption with human anti A (anti GalNAc) substance. They could, however, not support this conclusion by using glycosidases. In contrast to the findings of Morris *et al.* (1977), Isaacsson (1977) reported that the total carbohydrate content of the K99 adhesin isolated from K12 ($K99^+$) recombinant was only 0.6% and failed to detect the presence of either N-acetylglucosamine or N-acetylgalactosamine. Our experiments on the K99 adhesin (provided by Dr. J.A. Morris) showed that the total carbohydrate content was only 1.44% which suggests that these

low levels of carbohydrate represent simply contaminations of the protein adhesin molecule. Such contaminations may well arise from the cell wall of the bacterium during the process of isolation. With regard to N-acetylgalactosamine, its apparent concentration in the adhesin was 0.2%. Such a concentration is unlikely to account for the activity of the adhesin as relatively high concentrations of the free sugar were required before any inhibition of the haemagglutination or milk fat globule agglutination assays were observed. The loss of haemagglutination activity of the K99 adhesin following its treatment with periodate could be explained by oxidation of hydroxy amino acids which might be present in the K99 adhesin as suggested by Morris et al. (1977). As stated before, Spiro (1964) has stressed that the molar ratio of periodate:glycoprotein is critical if the specific destruction of carbohydrate is to be ensured, and recommended ratios of between 100:1 and 300:1 for this purpose. Ratios in excess of these values could lead to oxidation of protein residues. On the other hand, Westwood et al. (1976) showed that treatment of Carcinoemberyonic antigen with concentrations as low as 5 mM of periodate resulted in an undesired cleavage of the protein chain together with the destruction of 30 - 60% of tyrosine and 60% of tryptophan. It is accordingly difficult to attribute the loss of the haemagglutinating activity of the K99 adhesin to concomitant loss of carbohydrate alone. The position regarding the K88 adhesin is presently similar in that the K88 adhesin is regarded as a pure protein usually contaminated with carbohydrate (Stirm et al., 1967). The native filamentous structure of the K99 adhesin, its subunit structure and its physical location on the bacterial cell envelope are consistent with its being pilus or pilus-like structure (Isaacsson,1977). The pili are

elongated filamentous protein-containing structures that ride on the outside of the bacterial cell envelope (Brinton, 1965).

From the above discussion it can be concluded that the K99 adhesin of E. coli B41 is almost certainly a protein with some carbohydrate contamination. It can be assumed to have specific binding sites for carbohydrate determinants, containing N-acetylgalactosamine, on the surfaces of bovine milk fat globules and sheep erythrocytes and presumably also on the intestinal epiethlial membranes of the calf host. As the attachment of the K99 adhesin to bovine milk fat globules and sheep erythrocytes can be specifically inhibited by N-acetylgalactosamine, the K99 adhesin can be regarded as a lectin in accordance with the definition of lectins proposed by Goldstein et al., (1980) and Kocourek and Horejsi (1981).

The establishment of the bovine MFGM as a model system for studying the interaction of the K99 adhesin with the intestinal epithelial membranes should contribute to the state of our knowledge about the biochemical basis of the process of adhesion. The advantages of this model system over that of sheep erythrocytes can be summarised in two main points:

(i) Being from the same species, the bovine MFGM and calf brush border membranes are expected to have more common surface receptors than the sheep erythrocytes and calf brush border.

(ii) The bovine MFGM is an easily obtainable source that can be isolated in relatively pure form without cytoplasmic contaminations.

Suggestions for further work

(1) Isolation of plasma membranes from the mammary, epithelial cells would allow analytical comparison with the MFGM. The availability of antisera against whole MFGM and its isolated sialoglycopeptides would aid in the use of the comparative immunochemistry of the two membranes. Such examination would establish whether any major change in the surface components of the MFGM occurs during the process of milk secretion and would also provide more direct information about surface antigens of the mammary cell. Indeed, such information could help to identify any antigens that might be associated with disease such as breast carcinoma.

(2) Further studies are required to establish the glycosidic linkages of the carbohydrate moeities of the SP glycopeptide fraction and their contribution to the overall antigenicity of bovine MFGM, particularly in view of the evidence (Section A) that anti SP glycopeptide antisera and anti whole MFGM antisera showed similar specificites towards N-acetyl-D-glucosamine. The combination of data, derived by enzymic degradation with specific exoglycosidases coupled with periodate oxidation and methylation analysis, would result in the accurate proposal of the structure of the carbohydrate chains.

(3) Further studies are needed to establish any correlation, if any, between MFGMs from a range of different species and species-specific bacterial adhesins. These studies could be carried out as described in Section C.

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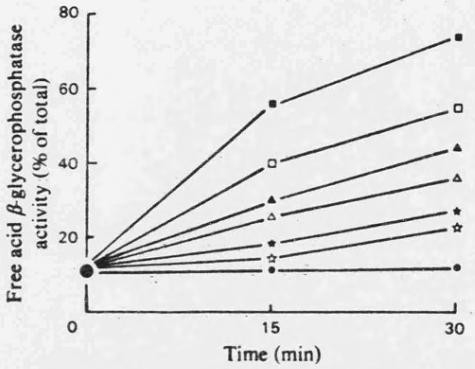

Fig. 1. *Increase in free acid β-glycerophosphatase of lysosomes incubated at 25°C in various test sugars at* 0.25M

The sugars tested were: ●, Sucrose; △, D-glucose; ▲, D-mannose; □, D-galactose; ■, D-ribose; ☆, L-glucose; and ★, D-glucose+1 mM-phloridzin.

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Presence of the Thomsen–Friedenreich Antigen on the Surface of Normal Human Milk Fat-Globule Membrane

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The Thomsen–Friedenreich antigen has been identified with the disaccharide β-D-galactopyranosyl-(1→3)-*N*-acetyl-D-galactosamine and its presence in masked (i.e. sialylated) form has been demonstrated on a number of mammalian membranes (Vaith & Uhlenbruck, 1978; Farrar & Harrison, 1978). Anti-(Thomsen–Friedenreich) antibodies are normally present in all human sera and the exposed Thomsen–Friedenreich antigen is not generally found on human cell surfaces. Springer *et al.* (1975), however, have reported that the Thomsen–Friedenreich antigen is exposed in breast carcinoma, but not in normal breast tissue and that concentrations of anti-(Thomsen–Friedenreich) antibodies are decreased in a relatively high percentage of cancer patients. In contrast, Klein *et al.* (1978) have used fluorescein- and ^{3}H-labelled peanut (*Arachis hypogaea*) agglutinin to show the present of exposed Thomsen–Friedenreich antigen on the surface of mammary epithelial cells of healthy individuals, and other studies (R. Newman, personal communication) have failed to detect significant differences in the anti-(Thomsen–Friedenreich) titres of sera from normal and from breast-cancer patients. We have made use of lectin-induced agglutination of intact milk fat-globules to provide evidence for the presence of receptors for peanut agglutinin on the surface of normal human milk fat-globule membranes.

The milk fat-globule membrane is generally accepted as being derived from the apical cell membrane, and probably also from the Golgi vesicle membrane, of the mammary secretory cell (Patton & Keenan, 1975; Powell *et al.*, 1977; Wooding, 1977). The presence of antigens on the surface of the milk fat-globule membrane may accordingly be taken as

evidence that such antigens are also present on the mammary cell membrane. Samples of human milk were taken from healthy individuals, including a range of ABO and MN blood types, and incubated with serial dilutions of the following lectins: *Lens culinaris*, *Lotus tetragonolobus*, *Helix pomatia*, *Triticum vulgaris*, *Ricinus communis*, *Ulex europeus*, peanut agglutinin, *Dolichos biflorus*, *Vicia graminea*. All the human milk samples tested were agglutinated by peanut agglutinin [specific for Thomsen–Friedenreich antigen (Lotan *et al.*, 1975)], *Ricinus communis* (galactose), *Lens culinaris* (mannose) and *Triticum vulgaris* (*N*-acetylglucosamine) at moderate dilutions and only poorly, or not at all, by the remaining lectins. In contrast with pooled bovine milk, which was agglutinated by moderate dilutions of most lectins tested, human milk was not rendered agglutinable by higher dilutions of peanut lectin after neuraminidase treatment. The results obtained with peanut lectin are consistent with the presence of the Thomsen–Friedenreich antigen in an exposed, but not sialylated, form on the surface of normal human milk fat-globule membrane. This has not previously been shown. Horisberger *et al.* (1977) used gold-labelled lectins and electron microscopy to demonstrate the presence of wheat germ agglutinin, soya bean agglutinin and concanavalin A receptors on human milk fat-globule membrane, but results with labelled peanut lectin were inconclusive. Glöckner *et al.* (1976) found chemical evidence for the presence of unsubstituted β-D-galactopyranosyl-(1 → 3)-*N*-acetyl-D-galactosamine in a glycoprotein fraction extracted from human milk fat-globule membranes although haemagglutination-inhibition techniques failed to show the presence of the Thomsen–Friedenreich antigen in any form in the same glycoprotein fraction (Newman & Uhlenbruck, 1977).

The present indication of the unsubstituted Thomsen–Friedenreich antigens on normal human milk fat-globules and, by implication, also on the mammary secretory cell membrane of healthy individuals supports the histochemical data of Klein *et al.* (1978) and accordingly questions the significance of the Thomsen–Friedenreich antigen as a tumour-associated antigen.

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Hydrogenation of the Polyunsaturated Lipids of Chloroplast Membranes and the Effects on Photosynthetic Functions

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Plant membranes, particularly those of the chloroplast, contain a high proportion of highly unsaturated lipids. Linolenic acid ($C_{18:3}$), for example, usually accounts for about 70% of the total fatty acyl residues associated with the galactolipids of higher-plant chloroplast lamellae (Douce *et al.*, 1973). It has been argued that a high proportion

SURFACE CARBOHYDRATES OF HUMAN MILK FAT GLOBULE MEMBRANE

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Milk fat globule membrane is derived from the apical membrane of the secretory cell and accordingly represents a convenient model system for structural and immunochemical studies of mammalian epithelial cell membranes.

The presence of specific monosacharide residues exposed on the outer surface of human milk fat globules was demonstrated by lectin-induced agglutination of washed globules. Thus galactose, N-acetyl-glucosamine and mannose were all detected by means of specific agglutination using ricinus, wheat germ agglutinin and lens lectins respectively.

The above sugars, together with N-acetylgalactosamine, sialic acid and fucose were released in the form of glycopeptides when washed human milk fat globules were incubated in the presence of Pronase. The sialoglycopeptides were fractionated by gel-filtration and ion-exchange chromatography to give apparently homogeneous fractions the sugar contents of which were analysed by gas-liquid chromatography. Alkaline borohydride treatment of purified sialoglycopeptides released sialic acid-containing oligosaccharides containing galactose and N-acetyl-galactosamine similar to those derived from bovine milk fat globule membrane (Farrar & Harrison, 1978).

The pronase-cleaved sialoglycopeptides obtained from human milk fat globule membrane were found to act as powerful inhibitors of a radioimmunoassay (Heyderman et al. (1979) designed to detect human epithelial cell membrane antigens. Application of this assay (in collaboration with Dr.M.G.Ormerod of the Ludwig Institute for Cancer Research) has allowed a preliminary characterization of the molecular structure of the antigenic determinants derived from the milk fat globule.

These studies not only provide a model system for studies of the molecular nature of cell surface antigens in general but also offer the possibility of defining antigens specific for epithelial cell membranes. Such antigens and their corresponding antibodies have potential application in clinical monitoring of patients suffering from breast cancer.

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COMPARISON OF LECTIN RECEPTORS ON THE SURFACE OF HUMAN AND BOVINE MILK FAT GLOBULE MEMBRANES

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Abstract—1. The accessibility of specific carbohydrate residues on the surfaces of intact bovine and human milk fat globules has been compared by means of a lectin-based agglutination assay.

2. The presence of galactose, mannose and/or glucose and *N*-acetyl-D-glucosamine, but not fucose was detectable on both bovine and human globules.

3. The major differences between bovine and human milk fat globules were firstly, the presence on human globules of high amounts of *Arachis hypogaea* receptor, none of which was apparently masked by sialic acid and secondly, the presence of unsubstituted *N*-acetyl-D-galactosamine on bovine but not on human milk fat globules.

INTRODUCTION

Cell surface carbohydrate groupings, both glycolipid and glycoprotein in nature, are known to be involved in a wide range of cellular recognition processes (Harrison & Lunt, 1980) and while considerable data have been accumulated concerning surface structures of blood cells, much less is known about the carbohydrate components of other mammalian cell membranes.

The milk fat globule is surrounded by a membrane (MFGM) that is derived from the apical plasma membrane of the mammary secretory cell (Patton & Keenan, 1975; Anderson & Cawston, 1975; Wooding, 1977) and expressed globules accordingly constitute a convenient and large scale source of epithelial mammalian membrane for structural and immunological studies. Thus, human MFGM has been used as a source of antigen believed to be specific for epithelial cells (Heyderman *et al.*, 1979) and HLA-DR-like antigens have been detected on human milk fat globules (Wiman *et al.*, 1979). Evidence has also been presented (Reiter & Brown, 1976) for the presence of specific gut epithelial cell membrane receptors for *E. coli* on the surface of bovine MFGM.

Structural studies of MFGM constituents have been largely concentrated on the bovine membrane. Thus sodium dodecyl sulphate-polyacrylamide gel analysis of bovine MFGM has indicated the presence of up to 8 glycoprotein components (Kobylka & Carraway, 1972; Anderson *et al.*, 1974; Shimizu *et al.*, 1976; Murray *et al.*, 1979) while certain major glycoproteins have been isolated from this source and chemically analysed (Basch *et al.*, 1976; Newman *et al.*, 1976; Keenan *et al.*, 1977; Snow *et al.*, 1977). More detailed compositional and structural studies of the carbohydrate components of bovine MFGM have resulted from proteolytic cleavage of glycopeptides from the surface of bovine milk fat globules followed by purification and analysis (Harrison *et al.*, 1975; Farrar & Harrison, 1978). Human MFGM has been very much less studied. Electrophoresis has been used to separate five lectin-receptor glycoproteins from the human membrane (Murray *et al.*, 1979) and the lectin-binding properties of a phenol-extracted glycoprotein mixture from the same source have been studied (Newman & Uhlenbruck, 1977). Preliminary carbohydrate analyses of glycopeptides cleaved by proteases from human MFGM have also been reported (Harrison *et al.*, 1979).

All the above-mentioned analytical studies have concerned glycoproteins extracted in various ways from fat globule membranes. Sugar residues exposed on the surface of intact fat globules may well be subjected to differential extraction or to conformational changes during purification of glycoprotein fractions while glycolipid components are largely lost. It is clear that the contribution of carbohydrate structures to specific interactions of the milk fat globule membrane and, by implication, also of the mammary epithelial cell membrane, can most directly be studied in the intact fat globule. Microelectrophoretic techniques have been applied (Newman & Harrison, 1973) in demonstrating the exposure of sialic acid on the surface of intact bovine milk fat globules but other sugars were not detectable by these means. More recently Horisberger *et al.*, (1977) in an interesting electron microscopic study have shown the ability of certain gold-labelled lectins to bind in a specific manner to the surfaces of human and bovine milk fat globules. We now report the results of a comparative study of the accessibility of various sugar residues on the surfaces of intact human and bovine milk fat globules carried out by using a quantitative assay involving lectin-induced agglutination of intact fat globules.

MATERIALS AND METHODS

Arachis hypogaea (peanut agglutinin), *Ricinus communis* (types I and II) *Lens culinaris*, concanavalin A, wheat germ agglutinin and *Lotus tetragonolobus* lectins were all obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Source plant material for Soya bean, *Ulex*

europeus and *Dolichos biflorus* lectins, extracted agglutinin from *Helix pomatia* and typed human red blood cells were generous gifts from Dr D. J. Anstee, South West Regional Blood Transfusion Centre, Southmead, Bristol. *Vicia graminea* seeds were kindly supplied by Dr W. Dahr, Department of Immunobiology, Medical University Clinic, Cologne. PBS tablets were obtained from Oxoid Ltd, Basingstoke, Hants., U.K.

Neuraminidase (*Vibria comma* (cholerae)) was purchased from Behringwerke, Marburg/Lahn, G.F.R. All other reagents were from BDH Chemicals Ltd., Poole, Dorset, U.K.

Fresh bovine milk was obtained from Friesian cows in mid-lactation and each sample was a pool from four individual animals. Human milk was from individual volunteers in established lactation.

Washed milk fat globules

Fresh milk (10 ml) was centrifuged at 1500 *g* for 10 min at 22°C. The separated milk fat globules were resuspended in phosphate-buffered saline, pH 7.3 (10 ml) and recentrifuged under the same conditions. The washing procedure was repeated a further three times after which the milk fat globules were resuspended in phosphate-buffered saline, pH 7.3, so as to give an optical density of approx 1.7 O.D. when read against water at 700 nm.

Neuraminidase treatment of milk fat globules

Washed milk fat globules (from 5 ml original milk) were suspended in phosphate-buffered saline, (1 ml), pH 5.6, containing 1 mM $CaCl_2$ and neuraminidase (50 μl, 1 i.u./ml) and the mixture was incubated at 37°C for 30 min. The treated globules were removed by centrifugation at 1500 *g* for 10 min and resuspended in phosphate-buffered saline, pH 7.3 (5 ml). The washing procedure was repeated a further three times after which the globules were resuspended in phosphate-buffered saline as described above.

Lectin-induced agglutination of milk fat globules

A suspension of washed milk fat globules (20 μl) prepared as described above was mixed with lectin solution (20 μl) and phosphate-buffered saline (20 μl) pH 7.3, in a recessed microscope slide well and the agglutination was allowed to proceed at 22°C for 1 hr in a humid environment. The fat globules were examined at 100× magnification using a light microscope. Agglutination titres are expressed as the reciprocal of the minimum dilution of lectin giving agglutination of less than 30% of the total fat globules.

Lectin solutions

Commercially-obtained lectins were dissolved in phosphate-buffered saline, pH 7.3, to an initial concentration of 2 mg/ml except in the case of *Lotus tetragonolobus* when 1 mg/ml was used. Samples were serially diluted (2×) with phosphate-buffered saline for agglutination assays.

For lectins extracted directly from plant material, dry source material (1 g) was ground in a mortar and added to phosphate-buffered saline, pH 7.3, (10 ml) at 4°C. The suspension was homogenized (MSE bench homogenizer, 6 × 30 sec) at 4°C and allowed to stand at 4°C for a further 5 min before centrifugation at 14,000 *g* for 10 min at 4°C. The aqueous supernatant was extracted (3 × 1 vol) with diethyl ether, separated and freed from residual ether in a stream of N_2 at 4°C.

The activities of all lectin solutions were checked by demonstrating haemagglutination of neuraminidase-treated type O, NN red blood cells (or native type A cells in the cases of *Helix pomatia* and *Dolichos biflorus*) by high dilutions of the lectin solution. Specificities were confirmed by showing complete inhibition of haemagglutination caused by four haemagglutination doses of lectin (i.e., by 16× less dilution than the haemagglutination titre) when a 10 mM solution of the immunodominant sugar (20 μl) was incubated with the lectin solution for 30 min at 22°C prior to addition of red blood cells in the haemagglutination assay. The specificity of agglutination of milk fat globules was also checked directly by similar inhibition of three agglutination doses of lectin by 30 mM solutions of the respective immunodominant sugars. In the case of *Arachis hypogaea* specificity of milk fat globule agglutination was demonstrated by inhibition of three agglutination doses of the lectin by a purified glycopeptide fraction (see below) containing approx 0.3 mM Thomsen–Friedenreich antigen (Farrar, 1978) or by 10 mM concentrations of purified Thomsen–Friedenreich antigen obtained by alkaline borohydride treatment of this peptide. The glycopeptide remaining after alkaline borohydride treatment (Farrar, 1978) did not show inhibition at concentrations up to 2 mg/ml.

Thomsen-Friedenreich antigen-rich glycopeptide

Sialoglycopeptides were cleaved from the surface of intact washed bovine milk fat globules and purified by gel filtration on Sephadex G-50 as described by Farrar & Harrison (1978). The sialoglycopeptide mixture was further fractionated by ion-exchange on DEAE-Sephadex when elution with a linear gradient of pyridine acetate gave a sialic acid-rich glycopeptide fraction (Farrar, 1978), carrying a high concentration of sialic acid-substituted Thomsen–Friedenreich antigens (Farrar & Harrison, 1978). The purified glycopeptide was freed from sialic acid by treatment with neuraminidase and gel filtration on Sephadex G25 (Farrar, 1978). The carbohydrate component of the resulting glycopeptide was largely composed of alkali-labile Thomsen–Friedenreich antigen together with smaller amounts of alkali-stable oligosaccharides.

Haemagglutination assays

Samples of human red blood cells (50% suspension in acid-citrate dextrose anti-coagulant) were diluted (to 10 ml) in phosphate-buffered saline, pH 7.3, and centrifuged at 3500 *g* for 10 min. The pelleted cells were resuspended in phosphate-buffered saline and again centrifuged. This procedure was repeated three times after which washed, pelleted cells were diluted (to 3% v/v) in phosphate-buffered saline, pH 7.3, before use in agglutination studies.

Neuraminidase-treated cells were prepared by suspension of pelleted native red blood cells (0.5 ml) in phosphate-buffered saline, pH 5.6, (4.5 ml) containing 1 mM $CaCl_2$ and neuraminidase (50 μl, 1 i.u./ml) and the mixture was incubated at 37°C for 30 min. Cells were freed from enzyme by repeated (3×) centrifugation at 3500 *g* for 10 min and resuspension in phosphate-buffered saline, pH 7.3 (10 ml). Final packed, washed cells were suspended (3% v/v) as above for agglutination studies.

For the haemagglutination assay, test cells prepared as above (20 μl suspension) were mixed with phosphate-buffered saline, pH 7.2, (20 μl) and lectin solution (20 μl) and allowed to stand in the well of a microtitre plate for 1 hr at 37°C. Absence of agglutination was indicated by the presence of a compact "button" of cells at the bottom of the well.

RESULTS AND DISCUSSION

Pooled bovine milk fat globules were found to be agglutinated by high dilutions of *Ricinus communis* (types I and II), soya bean and wheat germ agglutinins, concanavalin A and *Helix pomatia* lectin. In all cases agglutination was specifically inhibited by the corresponding monosaccharide receptor (Table 1). Specific agglutination by these lectins demonstrates

Table 1. Agglutination titres of human and bovine milk fat globules with different lectins

Lectin	Bovine milk fat globules*		Human milk fat globules†	
	Native, washed	Neuraminidase-treated	Native, washed	Neuraminidase-treated
Ricinus communis type I (galactose, *N*-acetylgalactosamine)	2^6	2^8	2^{10}	2^{10}
Ricinus communis type II (galactose)	2^7	2^9	2^8	2^8
Soya bean agglutinin (galactose)	2^7	2^9	2^5	2^7
Concanavalin A (mannose/glucose)	2^{4-5}	2^{7-8}	2^8	2^{10}
Lens culinaris (mannose/glucose)	2^1	2^{2-3}	2^8	2^{9-10}
Wheat germ agglutinin (*N*-acetylglucosamine)	2^8	2^8	2^8	2^{8-9}
Ulex europaeus (fucose)	neg.	neg.	neg.	neg.
Lotus tetragonolobus (fucose)	neg.	neg.	neg.	2^0
Arachis hypogaea (Thomsen–Friedenreich antigen)	2^{2-3}	2^{5-6}	2^{7-8}	2^{7-8}
Vicia graminea (in part Thomsen-Friedenreich antigen)	neg.	2^{6-7}	neg.	neg.
Helix pomatia (*N*-acetylgalactosamine)	2^9	2^{10}	neg.	2^3
Dolichos biflorus (*N*-acetylgalactosamine)	2^4	2^6	neg.	neg.

* Values are the means of titres obtained by using 5 samples of milk each of which was pooled from 4 individual cows.

† Values are the means of titres obtained by using 20 samples of milk all from different individuals of varying ABO and MN blood groups. No differences in titre were observed with different blood groups.

the accessibility of galactose (*Ricinus* types I and II, soya bean), mannose and/or glucose (concanavalin A), *N*-acetyl-D-glucosamine (wheat germ agglutinin), and *N*-acetyl-D-galactosamine (*Helix pomatia*) on the surfaces of intact fat globules. Our observations are entirely consistent with the results of mild proteolytic treatment of washed bovine milk fat globules which released, in soluble form, glycopeptides containing all these sugars together with sialic acid and fucose (Harrison *et al.*, 1975). Low concentrations (1 mg/ml) of a crude mixture of such glycopeptides purified on Sephadex G-50 (Farrar & Harrison, 1978) were found to inhibit strongly the agglutination of bovine milk fat globules by all the above-mentioned lectins.

Although the presence of fucose in bovine MFGM glycopeptides has been demonstrated by gas chromatography (Harrison *et al.*, 1975), this was not reflected in agglutination of the globules by either of the fucose-specific lectins, *Ulex europaeus* or *Lotus tetragonolobus*. Horisberger *et al.* (1977) were similarly unable to demonstrate marking of bovine milk fat globules by gold-labelled anti-H lectin and it appears that although fucose is normally a chain-terminal unit, access to the sugar is hindered not only in the case of gold-labelled lectins but also in that of the underivatized reagents.

In contrast to the findings of Horisberger *et al.* (1977), specific interaction of bovine milk fat globules with peanut agglutinin (*Arachis hypogaea*) could be demonstrated by means of the agglutination assay (Table 1). Peanut agglutinin has been shown (Lotan *et al.*, 1975; Pereira *et al.*, 1976) to have high affinity for the Thomsen-Friedenreich antigen, β-D-galactopyranosyl-(1-3)-*N*-acetyl-D-galactosamine, and the involvement of this dissacharide in the Arachis-induced agglutination of bovine globules was further indicated by the fact that agglutination could be inhibited by low concentrations of a purified glycopeptide (Materials and Methods) rich in unsubstituted Thomsen–Friedenreich antigen (0.3 mM disaccharide). A tetrasaccharide and two trisaccharides containing sialic acid-substituted Thomsen–Friedenreich antigen have been fully characterized (Farrar & Harrison, 1978) in bovine MFGM-derived glycopeptides. The unsubstituted disaccharide was not specifically sought in these studies but has been detected (Newman *et al.*, 1976) by haemagglutination-inhibition techniques in a phenol-extracted glycoprotein fraction from bovine MFGM. Our present evidence for the exposure of unmasked Thomsen-Friedenreich antigen on the surface of bovine milk fat globules clearly complements these latter findings and indicates that the presence of unsubstituted disaccharide in extracted glycoproteins does not result from artefactual cleavage of sialic acid residues during the extraction procedure. The failure of Horisberger *et al.* (1977) to mark bovine fat globules with gold-labelled peanut lectin could reflect a more restricted access of the bulky labelled reagent to the surface receptors.

As might be expected, pre-treatment of bovine milk

fat globules with neuraminidase increased the Arachis-induced agglutination titre, consistent with the cleavage of sialic acid residues from the surface tri- and tetrasaccharides (Farrar & Harrison, 1978) with consequent exposure of the Thomsen–Friedenreich antigenic core. It is of interest that *Vicia graminea*, which is known (Uhlenbruck & Dahr, 1971) to require the presence of Thomsen–Friedenreich antigen in its specific receptor site, only causes agglutination of bovine globules after neuraminidase treatment of the globules when the concentration of exposed disaccharide is increased.

The presence of *N*-acetyl-D-galactosamine in an exposed form on the surface of bovine milk fat globules was shown by the specific agglutinations induced by *Helix pomatia* and *Dolichos biflorus* lectins. This supports previous reports (Newman *et al.*, 1976; Newman & Uhlenbruck, 1977) that phenol-extracted glycoproteins from bovine MFGM carry unsubstituted N-acetyl-D-galactosamine. The agglutination titre was slightly increased by neuraminidase treatment of the bovine globules.

Human MFGM is less readily available than the bovine membrane and has been correspondingly less studied. Human milk fat globules were specifically agglutinated by high dilutions of *Ricinus communis* (type I & II), soya bean, concanavalin A, Lens and wheat germ agglutinins, demonstrating the accessibility of galactose, (Ricinus, soya bean agglutinin) mannose and/or glucose (concanavalin A, Lens lectins) and *N*-acetyl-D-glucosamine (wheat germ agglutinin) on the surfaces of the intact fat globules. Agglutination of human globules by these lectins is generally similar to that of bovine globules although titres, particularly for concanavalin A and Lens lectins were higher in the human case (Table 1). Our results support electron microscopic data in which human milk fat globules were found to be marked by gold-labelled soya bean and wheat germ agglutinins and, although to a lesser extent, by concanavalin A (Horisberger *et al.*, 1977). The ability of intact human milk fat globules to bind soya bean agglut in contrasts with the behaviour of extracted huma MFGM glycopeptides which, unlike the corresponding bovine extracts, interact weakly (Newman & Uhlenbruck, 1977) or not at all (Murray *et al.*, 1979) with soya bean lectin. It may be that soya bean lectin-induced agglutination of intact human milk fat globules and marking of the globules by gold-labelled soya bean lectin (Horisberger *et al.*, 1977) involve exclusively glycolipid-bound galactose. The increased interctions (Table and Horisberger *et al.*, 1977) observed following neuraminidase treatment of the globules suggest, however, that glycoprotein-bound galactose is involved at least in the response of sialic acid-free globules to soya bean lectin because, as Horisberger *et al.* (1977) have pointed out, MFGM proteins mask membrane gangliosides from neuraminidase attack (Tomich *et al.*, 1976).

As with bovine globules, human milk fat globules were not agglutinated by the fucose-specific lectins, *Ulex europaeus* and *Lotus tetragonolobus* although weak agglutination was detected with the latter lectin following neuraminidase treatment of the human globules. Most lectins do, in fact, show increased agglutination titres after treatment of either bovine or human fat globules with neuraminidase. It is likely that such increases generally reflect unmasking of relevant sugar receptors as a consequence of the removal of terminal sialic acid residues. It could be that enhanced agglutinability under such circumstances simply arises from non-specific aggregation resulting from loss of surface negative charges although the unchanged Arachis-agglutination titres of human fat globules following neuraminidase treatment argue against this.

The strong agglutination of human milk fat globules by peanut agglutinin (Table 1) has been preliminarily reported (Farrar *et al.*, 1979). As in the case of bovine fat globules, agglutination was specifically inhibited by low concentrations (0.5 mg/ml) of a purified glycopeptide rich in Thomsen–Friedenreich antigen. (Materials and Methods.) Agglutination was also strongly inhibited by 10 mM Thomsen–Friedenreich antigen from the above glycopeptide but not by the residual glycopeptide (2 mg/ml) from which the disaccharide had been removed (Materials and Methods). The exposure of unsubstituted Thomsen–Friedenreich antigen on the surface of intact human milk fat globules has not previously been demonstrated, although Klein *et al.* (1978) have provided evidence for exposure of the antigen on the surface of normal mammary epithelial cells from which the globule membrane is derived. These results are contrary to those of Springer *et al.* (1975) who found the Thomsen–Friedenreich antigen to be exposed on breast carcinoma but not in normal tissue. As is the case with soya bean agglutinin, peanut lectin apparently behaves differently with extracted human MFGM glycoproteins. Thus, Newman & Uhlenbruck (1977) were unable to show inhibition of peanut lectin-induced agglutination of desialylated erythrocytes either by native or by desialylated human MFGM glycoproteins indicating that the Thomsen-Freidenreich antigen was not accessible on their glycoprotein extract, despite the fact that it was detected chemically in the same fraction (Glöckner *et al.*, 1976).

As mentioned above, human fat globules were not rendered more agglutinable by peanut lectin following their treatment with neuraminidase. This is in contrast to the behaviour of bovine milk fat globules and suggests that unlike the latter, human globules do not carry appreciable amounts of sialylated Thomsen–Friedenreich antigen in tri- and tetrasaccharide form (Farrar & Harrison, 1978) exposed on their outer surface. These conclusions are supported by the results obtained using *Vicia graminea* lectin which failed to agglutinate human fat globules even after neuraminidase treatment of the globules. Under similar circumstances the Thomsen–Friedenreich antigens of bovine milk fat globules were apparently unmasked sufficiently to allow agglutination by high dilutions of *Vicia graminea* lectin, even though, as in the human case, native globules were not agglutinated by the highest concentrations of lectin.

In contrast to bovine globules, human milk fat globules were agglutinated neither by *Helix pomatia* nor by *Dolichos biflorus* lectins irrespective of the blood group of the human donor. It is of interest that donors of A-blood group specificity express the A antigen, *N*-acetyl-D-galactosamine, on their red blood

cells (by definition) but not apparently on milk fat globules. The opposite is true of the bovine system in which unsubstituted *Helix pomatia* receptor has been detected on intact milk fat globules (Table 1) but not on erythrocytes (Kim & Uhlenbruck, 1966). Results obtained from membrane-derived glycoprotein fractions agree with these findings in the bovine (Newman *et al.*, 1976; Newman & Uhlenbruck, 1977) but not in the human case, when *Helix pomatia* receptors were detected on phenol-extracted glycoprotein fractions from human MFGM (Glockner *et al.*, 1976).

The milk fat globule agglutination assay employed in this study is a simple technique capable of rapidly providing quantitative information about the accessibility of a wide range of membrane-bound sugar components without the need for chemical devivatization. The method is applicable to surface antigens in general and has been used to define immunodominant groupings of MFGM by hapten inhibition studies using anti- (bovine MFGM) antisera (Farrar & Mohanna, unpublished results).

Insofar as our findings overlap with the elegant but more limited studies of Horisberger *et al.*, 1977, using gold-labelled lectins, similar results were largely obtained by the two methods and occasional differences (e.g. response to peanut lectin) can be explained in terms of hindered access of the relatively-bulky labelled lectins to the globule surface. Differences between interactions of lectins with intact globules on the one hand and with isolated glycoproteins on the other, are more marked and could well reflect changes resulting from extraction in the latter case. It may also be that glycolipids contribute significantly to the specific interactions of the MFGM surface.

One of the most significant differences between human and bovine MFGM found by these agglutination studies is the lack of effect of neuraminidase on peanut lectin-induced agglutination of human globules suggesting that the latter do not carry significant amounts of the tri- and tetrasaccharide residues which have been isolated from bovine globules (Farrar & Harrison, 1978). These oligosaccharides have been characterized in membranes of human erythrocytes (Thomas & Winzler, 1969), rat brain (Finne, 1975) and on other membranes (Krusius & Finne, 1977) and their apparent absence from human milk fat globules raises the problem of their function generally. A second major difference concerns the presence of *Helix pomatia* receptors on bovine but not on human milk fat globules despite the A-blood group specificity of many of the human donors. It is an intriguing fact that in both these cases the structure present on bovine but not on human globules has been reported in human but not in bovine erythrocyte membranes (Kim & Uhlenbruck, 1966; Thomas & Winzler, 1969; Emerson & Kornfeld, 1976).

The contributions of MFGM lectin receptors to specific interactions of the milk fat globule, or more significantly, of the mammary epithelial cell membrane, remain to be elucidated, but it is likely that these exposed sugar residues will be shown to play an important role in recognition processes involving mammary cells or epithelial cells generally.

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